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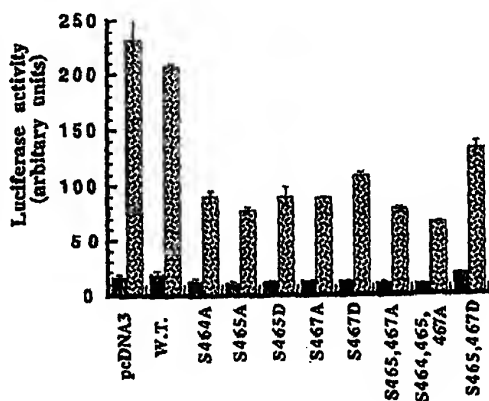
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(54) Title: SMAD2 PHOSPHORYLATION AND INTERACTION WITH SMAD4



(57) Abstract

The invention describes amino acid residues of the Smad2 protein which are important for phosphorylation activity, and Smad2 polypeptide fragments and biologically functional variants thereof. Included are dominant-negative variants of Smad2 and antibodies relating thereto. Also included are nucleic acids which encode such variants. Antibodies which selectively bind pathway-restricted Smad proteins phosphorylated at the C-terminal tail also are provided. Methods and products for using such nucleic acids and polypeptides also are provided.

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SMAD2 PHOSPHORYLATION AND INTERACTION WITH SMAD4**Field of the Invention**

This invention relates to nucleic acids and encoded polypeptides which interact with the
5 TGF- β receptor complex and which is a negative regulator of TGF- β signaling. The invention
also relates to agents which bind the nucleic acids or polypeptides. The invention further relates
to methods of using such nucleic acids and polypeptides in the treatment and/or diagnosis of
disease.

Background of the Invention

10 During mammalian embryogenesis and adult tissue homeostasis transforming growth
factor β (TGF- β) performs pivotal tasks in intercellular communication (Roberts et al., *Growth
Factors*, 8:1-9, 1993). The cellular effects of this pleiotropic factor are exerted by ligand-
induced hetero-oligomerization of two distantly related type I and type II serine/threonine kinase
receptors, T β R-I and T β R-II, respectively (Lin and Lodish, *Trends Cell Biol.*, 11:972-978, 1993;
15 Derynck, *Trends Biochem. Sci.*, 19:548-553, 1994; Massague and Weis-Garcia, *Cancer Surv.*
27:41-64, 1996; ten Dijke et al., *Curr. Opin. Cell Biol.*, 8:139-145, 1996). The two receptors,
which both are required for signaling, act in sequence; T β R-I is a substrate for the constitutively
active T β R-II kinase (Wrana et al., *Nature*, 370:341-347, 1994; Wieser et al., *EMBO J.*,
14:2199-2208, 1995). TGF- β forms part of a large family of structurally related proteins which
20 include activins and bone morphogenetic proteins (BMPs) that signal in a similar fashion, each
employing distinct complexes of type I and type II serine/threonine kinase receptors (Lin and
Lodish, 1993; Derynck, 1994; Massague and Weis-Garcia, 1996; ten Dijke et al., 1996).

Genetic studies of TGF- β -like signaling pathways in *Drosophila* and *Caenorhabditis*
elegans have led to the identification of *mothers against dpp* (*Mad*) (Sekelsky et al., *Genetics*,
25 139:1347-1358, 1995) and *sma* (Savage et al., *Proc. Natl. Acad. Sci. USA*, 93:790-794, 1996)
genes, respectively. The products of these related genes perform essential functions downstream
of TGF- β -like ligands acting via serine/threonine kinase receptors in these organisms (Wiersdorf
et al., *Development*, 122:2153-2163, 1996; Newfeld et al., *Development*, 122:2099-2108, 1996;
Hoodless et al., *Cell*, 85:489-500, 1996). Vertebrate homologs of *Mad* and *sma* have been
30 termed *Smads* (Derynck et al., *Cell*, 87:173, 1996) or MADR genes (Wrana and Attisano, *Trends
Genet.*, 12:493-496, 1996). Genetic alterations in *Smad2* and *Smad4/DPC4* have been found in
specific tumor subsets, and thus *Smads* may function as tumor suppressor genes (Hahn et al.,

Science, 271:350-353, 1996; Riggins *et al.*, *Nature Genet.*, 13:347-349, 1996; Eppert *et al.*, *Cell*, 86:543-552, 1996). Smad proteins share two regions of high similarity, termed MH1 and MH2 domains, connected with a variable proline-rich sequence (Massague, *Cell* 85:947-950, 1996; Derynck and Zhang, *Curr. Biol.*, 6:1226-1229, 1996). The C-terminal part of Smad2, when
5 fused to a heterologous DNA-binding domain, was found to have transcriptional activity (Liu *et al.*, *Nature*, 381:620-623, 1996; Meersseman *et al.*, *Mech. Dev.*, 61:127-140, 1997). The intact Smad2 protein when fused to a DNA-binding domain, was latent, but transcriptional activity was unmasked after stimulation with ligand (Liu *et al.*, 1996).

Different Smads specify different responses using functional assays in *Xenopus*. Whereas
10 Smad1 induces ventral mesoderm, a BMP-like response, Smad2 induces dorsal mesoderm, an activin/TGF- β -like response (Graff *et al.*, *Cell*, 85:479-487, 1996; Baker and Harland, *Genes & Dev.*, 10:1880-1889 1996; Thomsen, *Development*, 122:2359-2366, 1996). Upon ligand stimulation Smads become phosphorylated on serine and threonine residues; BMP stimulates Smad1 phosphorylation, whereas TGF- β induces Smad2 and Smad3 phosphorylation (Hoodless
15 *et al.*, 1996; Liu *et al.*, 1996; Eppert *et al.*, 1996; Lechleider *et al.*, *J. Biol. Chem.*, 271:17617-17620, 1996; Yingling *et al.*, *Proc. Natl. Acad. Sci. USA*, 93:8940-8944, 1996; Zhang *et al.*, *Nature*, 383:168-172, 1996; Macías-Silva *et al.*, *Cell*, 87:1215-1224, 1996; Nakao *et al.*, *J. Biol. Chem.*, 272:2896-2900, 1996).

Smad4 is a common component of TGF- β , activin and BMP signaling (Lagna *et al.*,
20 *Nature*, 383:832-836, 1996; Zhang *et al.*, *Curr. Biol.*, 7:270-276, 1997; de Winter *et al.*, *Oncogene*, 14:1891-1900, 1997). Smad4 phosphorylation has thus far been reported only after activin stimulation of transfected cells (Lagna *et al.*, 1996). After stimulation with TGF- β or activin Smad4 interacts with Smad2 or Smad3, and upon BMP challenge a heteromeric complex of Smad4 and Smad1 has been observed (Lagna *et al.*, 1996). Upon ligand stimulation, Smad
25 complexes translocate from the cytoplasm to the nucleus (Hoodless *et al.*, 1996; Liu *et al.*, 1996; Baker and Harland, 1996; Macías-Silva *et al.*, 1996), where they, in combination with DNA-binding proteins, may regulate gene transcription (Chen *et al.*, *Nature*, 383:691-696, 1996).

Summary of the Invention

30 The invention provides isolated Smad2 polypeptides and agents which bind such polypeptides, including antibodies. The invention also provides isolated nucleic acid molecules which encode the foregoing polypeptides, unique fragments of those molecules, expression

vectors containing the foregoing, and host cells transfected with those molecules. The foregoing can be used in the diagnosis or treatment of conditions characterized by TGF- β signal transduction. The invention also provides methods for identifying pharmacological agents useful in the diagnosis or treatment of such conditions. Here, the identification of Smad2 amino acid residues phosphorylated *in vivo* is reported.

According to one aspect of the invention, an isolated Smad2 polypeptide is provided. The polypeptide has the amino acid sequence of SEQ ID NO:2 or its human homolog except that the polypeptide includes a mutation comprising a non-serine amino acid located at one or more of amino acids 464, 465 and 467. In certain embodiments, the isolated Smad2 polypeptide comprises a mutation which is located at a position or positions selected from the group consisting of 464; 465; 467; 464 and 465; 464 and 467; 465 and 467; and 464, 465 and 467. Preferably the isolated Smad2 polypeptide comprises a mutation or mutations of the serine residues to alanine residues (e.g., Ser465A) or aspartic acid residues (e.g., Ser465D) such as those selected from the group consisting of Ser464A; Ser465A; Ser467A; Ser464A and Ser465A; Ser464A and Ser467A; Ser465A and Ser467A; Ser464A, Ser465A and Ser465A; Ser465D; Ser467D; and Ser465D and Ser467D.

In other embodiments, the foregoing isolated polypeptide consists of a fragment or variant of the foregoing which retains the activity of the foregoing.

According to still another aspect of the invention, nucleic acid molecules which encode the foregoing polypeptides are provided. The nucleic acids can be composed of natural and/or non-natural nucleotides and linked with natural and/or non-natural internucleoside bonds.

According to still another aspect of the invention, the invention involves expression vectors, and host cells transformed or transfected with such expression vectors, comprising the nucleic acid molecules described above.

According to another aspect of the invention, there are provided isolated Smad4 binding polypeptides comprising the amino acid sequence of SEQ ID NO:3, which selectively bind a Smad4 protein or fragment thereof, provided that the isolated polypeptide is not wild type Smad2. In certain embodiments, the isolated Smad4 binding polypeptide comprises the C-terminal 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids of SEQ ID NO:2. Preferably, the isolated Smad4 binding polypeptide of comprises the amino acid sequence of SEQ ID NO:4. In other preferred embodiments, the foregoing isolated Smad4 binding polypeptides are phosphorylated on one or more amino acids selected from the group consisting

of Ser464, Ser465, Ser467, Ser464/Ser465, Ser464/Ser467, Ser465/Ser467 and Ser464/Ser465/Ser467.

According to yet another aspect of the invention, the invention involves an isolated polypeptide which binds selectively any of the foregoing isolated polypeptides, provided that the isolated polypeptide is not T β R-I, T β R-II or Smad4. In preferred embodiments, the isolated binding polypeptides include antibodies and fragments of antibodies (e.g., Fab, F(ab)₂, Fd and antibody fragments which include a CDR3 region which binds selectively to the polypeptides of the invention). Particularly preferred antibodies include monoclonal antibodies.

In another aspect of the invention, an isolated polypeptide is provided which binds selectively to at least one pathway-restricted Smad polypeptide or fragment thereof having the C-terminal amino acid sequence of SEQ ID NO:5. The pathway-restricted Smad polypeptide or fragment thereof is phosphorylated on at least one serine residue of SEQ ID NO:5, and the isolated polypeptide is not T β R-I, T β R-II or Smad4. In certain embodiments, the pathway restricted Smad polypeptide is selected from the group consisting of Smad1, Smad3, Smad5 and Smad9. In other embodiments, the at least one serine residue is selected from the group consisting of the second serine residue, the third serine residue and the second and third serine residues. In preferred embodiments, the isolated binding polypeptides include antibodies and fragments of antibodies (e.g., Fab, F(ab)₂, Fd and antibody fragments which include a CDR3 region which binds selectively to pathway-restricted Smad polypeptides). Particularly preferred antibodies include monoclonal antibodies. In certain embodiments, the antibody or antibody fragment binds selectively to one of the pathway restricted Smad polypeptides.

According to still another aspect of the invention, methods for inhibiting TGF- β signal transduction in a mammalian cell are provided. The methods involve contacting a mammalian cell with an amount of an inhibitor of phosphorylation of endogenous Smad2 effective to reduce TGF- β signal transduction in the mammalian cell. In certain embodiments of the foregoing methods, the inhibitor is a dominant negative Smad2 polypeptide, such as the foregoing Smad2 polypeptides which include a mutation at one or more of residues 464, 465 and 467.

The invention in still another aspect provides compositions comprising a Smad2 polypeptide which includes a mutation at one or more of residues 464, 465 and 467, and a pharmaceutically acceptable carrier.

The invention in a further aspect involves a method for decreasing TGF- β signal transduction activity in a subject. An agent that selectively binds to a TGF- β receptor and blocks

TGF- β signaling is administered to a subject in need of such treatment, in an amount effective to decrease Smad2 TGF- β signal transduction activity in the subject. Preferred agents are Smad2 polypeptides which include a mutation at one or more of serines 464, 465 and 467, particularly those which are substituted with alanines or aspartic acids.

- 5 According to another aspect of the invention, methods are provided for identifying lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated with Smad2/TGF- β receptor interaction. The methods involve forming a mixture of a Smad2 polypeptide comprising a mutation at one or more of serines 464, 465 and 467, a TGF- β receptor, and a candidate pharmacological agent. The mixture is incubated under conditions
- 10 which, in the absence of the candidate pharmacological agent, permit a first amount of specific binding of the TGF- β receptor by the Smad2 polypeptide. A test amount of the specific binding of the TGF- β receptor by the Smad2 polypeptide then is detected. Detection of a reduction in the foregoing activity in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which disrupts
- 15 the Smad2/TGF- β receptor interaction. Detection of an increase in the foregoing activities in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which enhances Smad2/TGF- β receptor interaction. Preferably the Smad2 polypeptide comprises a mutation or mutations selected from the group consisting of Ser464A; Ser465A; Ser467A; Ser464A and Ser465A; Ser464A and
- 20 Ser467A; Ser465A and Ser467A; Ser464A, Ser465A and Ser465A; Ser465D; Ser467D; and Ser465D and Ser467D.

- According to a further aspect of the invention, methods are provided for identifying lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated with TGF- β -mediated Smad2 signal transduction activity. The methods involve forming a
- 25 mixture of a wild type Smad2 polypeptide, a TGF- β receptor, and a candidate pharmacological agent comprising a mutated Smad2 polypeptide, preferably having a mutation at one or more of serines 464, 465 and 467. The mixture is incubated under conditions which, in the absence of the candidate pharmacological agent, permit a first amount of TGF- β mediated phosphorylation of the wild type Smad2 polypeptide. A test amount of TGF- β mediated phosphorylation of the
- 30 wild type Smad2 polypeptide then is detected. Detection of a reduction in the test amount of TGF- β mediated phosphorylation of the wild type Smad2 polypeptide relative to the first amount of TGF- β mediated phosphorylation of the wild type Smad2 polypeptide indicates that the

candidate pharmacological agent is a lead compound for a pharmacological agent which disrupts the TGF- β mediated Smad2 signal transduction activity. Preferably the mutated Smad2 polypeptide comprises a mutation or mutations selected from the group consisting of Ser464A; Ser465A; Ser467A; Ser464A and Ser465A; Ser464A and Ser467A; Ser465A and Ser467A; 5 Ser464A, Ser465A and Ser467A; Ser465D; Ser467D; and Ser465D and Ser467D, or TGF- β receptor binding fragments thereof.

According to another aspect of the invention, methods for determining the amount of a pathway-restricted Smad polypeptide having a phosphorylated C-terminal serine residue in a biological sample are provided. The methods include contacting the biological sample with an 10 isolated polypeptide that selectively binds at least one phosphorylated serine of the amino acid sequence as set forth in SEQ ID NO:5, determining the binding of the isolated polypeptide to the pathway-restricted Smad polypeptide, and comparing the binding to a control as a determination of the amount of a pathway-restricted Smad polypeptide having a phosphorylated C-terminal serine residue in the biological sample. The biological sample can be a biological extract such as 15 *in vitro* extract, or can be tissue sample for *in vivo* or *in vitro* immunohistochemistry analysis. In certain embodiments, the pathway-restricted Smad polypeptide is selected from the group consisting of Smad1, Smad2, Smad3, Smad5, and Smad9. In preferred embodiments, the isolated binding polypeptides include antibodies and fragments of antibodies, including monoclonal antibodies. In certain of the preferred embodiments, the antibody or antibody 20 fragment selectively binds a pathway-restricted Smad polypeptide having at least one phosphorylated C-terminal serine residue selected from the group consisting of the second serine residue of SEQ ID NO:5, the third serine residue of SEQ ID NO:5 and the second and third serine residues of SEQ ID NO:5.

According to yet another aspect of the invention, methods for identifying lead compounds 25 for a pharmacological agent which modulate phosphorylation of the C-terminal serine residues of a pathway-restricted Smad polypeptide are provided. The methods include forming a mixture comprising a pathway-restricted Smad polypeptide having at least one C-terminal serine residue, a TGF- β superfamily receptor or receptor complex capable of phosphorylating the at least one C-terminal serine residue, and a candidate pharmacological agent. The mixture is incubated under 30 conditions which, in the absence of the candidate pharmacological agent, permit a first amount of phosphorylation of the at least one C-terminal serine residue by the TGF- β superfamily receptor or receptor complex. A test amount of the phosphorylation of the at least one C-terminal serine

residue by the TGF- β superfamily receptor or receptor complex then is detected. A reduction of the test amount of phosphorylation relative to the first amount of phosphorylation indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces phosphorylation of the at least one C-terminal serine residue of a pathway-restricted Smad polypeptide. An increase of the test amount of phosphorylation relative to the first amount of phosphorylation indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases the phosphorylation of the C-terminal serine residues of a pathway-restricted Smad polypeptide. In certain embodiments, the pathway-restricted Smad polypeptide is selected from the group consisting of Smad1, Smad2, Smad3, Smad5, and Smad9.

10 In other embodiments, the at least one C-terminal serine residue is selected from the group consisting of the second serine residue of SEQ ID NO:5, the third serine residue of SEQ ID NO:5 and the second and third serine residues of SEQ ID NO:5. In still other embodiments, the step of forming a mixture comprising a pathway-restricted Smad polypeptide, a TGF- β superfamily receptor or receptor complex, and a candidate pharmacological agent includes

15 contacting a cell which includes a pathway-restricted Smad polypeptide and a TGF- β receptor or receptor complex with a candidate pharmacological agent. In preferred embodiments of the foregoing methods, the step of forming a mixture further comprises adding a ligand which activates the TGF- β superfamily receptor or receptor complex.

In another aspect of the invention methods for reducing heteromeric Smad protein

20 complex formation in a cell is provided. The methods include providing an antibody which selectively binds a phosphorylated pathway-restricted Smad polypeptide or a nucleic acid encoding the antibody, and contacting the cell with an amount of the antibody or the nucleic acid encoding the antibody sufficient to reduce formation of the heteromeric Smad protein complex in the cell. In certain embodiments, the pathway-restricted Smad polypeptide is selected from the

25 group consisting of Smad1, Smad2, Smad3, Smad5, and Smad9. In other embodiments, the antibody selectively binds a pathway-restricted Smad polypeptide having at least one phosphorylated C-terminal serine residue selected from the group consisting of the second serine residue of SEQ ID NO:5, the third serine residue of SEQ ID NO:5 and the second and third serine residues of SEQ ID NO:5.

30 Use of the foregoing compounds in the preparation of a medicament also is provided.

These and other aspects of the invention will be described in further detail in connection with the detailed description of the invention.

Brief Description of the Figures

Fig. 1 depicts T β R-mediated phosphorylation of Smad2.

Fig. 2 shows the identification of Ser465 and Ser467 as the major *in vivo* phosphorylation sites in Smad2.

5 Fig. 3 demonstrates that phosphorylation of Ser465 requires that Ser467 is phosphorylated.

Fig. 4 shows the dominant-negative effect on TGF- β -mediated transcriptional response of Smad2 mutants with serine residues 464, 465 and/or 467 replaced with alanine or aspartic acid residues.

10 Fig. 5 demonstrates the association of wild-type and mutant Smad2 with T β R-I.

Fig. 6 shows that phosphorylated peptides derived from the C-terminus of Smad2 bind to Smad4.

Fig. 7 demonstrates the specificity of a Smad2 phospho tail antibody for phosphorylated Smad2.

15 Fig. 8 shows that the Smad2 phospho tail antibody recognizes Smad2 phosphorylated in TGF- β stimulated cells.

Brief Description of the Sequences

SEQ ID NO:1 is the nucleotide sequence of the mouse Smad2 cDNA (accession number
20 U60530).

SEQ ID NO:2 is the amino acid sequence of the mouse Smad2 protein (accession number U60530).

SEQ ID NO:3 is the amino acid sequence of the four amino acid fragment of the Smad2 C-terminus which binds Smad4.

25 SEQ ID NO:4 is the amino acid sequence of the fourteen amino acid fragment of the Smad2 C-terminus which binds Smad4.

SEQ ID NO:5 is the amino acid sequence of the C-terminal motif of the pathway-restricted Smads.

30 SEQ ID NO:6 is the amino acid sequence of a peptide used to prepare polyclonal antibodies to phosphorylated Smad2.

SEQ ID NO:7 is the amino acid sequence of another phosphorylated Smad2 peptide used for preparation of antibodies.

SEQ ID NO:8 is the amino acid sequence of the C-terminus of Smad1, Smad3, Smad5 and Smad9 proteins.

SEQ ID NO:9 is the amino acid sequence of the phosphorylated peptide used for preparation of antibodies to Smad1, Smad3, Smad5 and Smad9.

5 SEQ ID NO:10 is the amino acid sequence of the fourteen amino acid fragment of the Smad1 protein C-terminus.

SEQ ID NO:11 is the amino acid sequence of the fourteen amino acid fragment of the Smad3 protein C-terminus.

10 SEQ ID NO:12 is the amino acid sequence of the fourteen amino acid fragment of the Smad5 protein C-terminus.

SEQ ID NO:13 is the amino acid sequence of the fourteen amino acid fragment of the Smad9 protein C-terminus.

SEQ ID NO:14 is the nucleotide sequence of the human Smad2 mRNA (accession number AF027964).

15 SEQ ID NO:15 is the amino acid sequence of the human Smad2 protein (accession number AF027964).

Detailed Description of the Invention

We present the identification of TGF- β -mediated phosphorylation of Smad2 at two serine
20 residues in the C-terminus of the protein, i.e., Ser465 and Ser467. Phosphorylation of Ser465 required that Ser467 was phosphorylated. Mutation of Ser465 and/or Ser467 in Smad2 to alanine residues resulted in dominant negative inhibition of TGF- β signaling, as did mutation of Ser464 which is not an *in vivo* phosphorylation site. These Smad2 mutants were found to interact stably with an activated TGF- β receptor complex, in contrast to wild-type Smad2, which
25 interacts only transiently. A peptide containing the four C-terminal amino acid residues of Smad2 with Ser465 and Ser467 phosphorylated, bound *in vitro* to a glutathione S-transferase-Smad4 fusion protein with higher affinity than the corresponding non-phosphorylated peptide. Binding to GST-Smad4 was strongly enhanced when a longer C-terminal peptide (14 amino acid residues) was used, and binding was in this case seen also in the absence of phosphorylation.

30 The invention thus involves in one aspect Smad2 polypeptides, genes encoding those polypeptides, functional modifications and variants of the foregoing, useful fragments of the foregoing, as well as therapeutics relating thereto. The invention also involves the recognition

that pathway-restricted Smad proteins (including Smad1, Smad2, Smad3, Smad5, and Smad9) share a conserved sequence at the C-terminus (SEQ ID NO:5), and therefore the invention relates to all of the pathway-restricted Smad proteins.

The invention provides isolated polypeptides, such as variants of the Smad2 polypeptides described previously (e.g., Nakao *et al.*, 1996; SEQ ID NO:2). In particular, mutants of the Smad2 polypeptide (e.g. the mouse protein is represented by SEQ ID NO:2) and fragments thereof are provided. Such polypeptides are useful, for example, alone for the modulation of TGF- β signal transduction or as fusion proteins to generate antibodies, e.g., for use as components of an immunoassay.

The peptides of the invention are isolated peptides. As used herein, with respect to peptides, the term "isolated peptides" means that the peptides are substantially pure and are essentially free of other substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. In particular, the peptides are sufficiently pure and are sufficiently free from other biological constituents of their hosts cells so as to be useful in, for example, producing pharmaceutical preparations or sequencing. Because an isolated peptide of the invention may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the peptide may comprise only a small percentage by weight of the preparation. The peptide is nonetheless substantially pure in that it has been substantially separated from the substances with which it may be associated in living systems.

As used herein, Smad2 polypeptides include polypeptides which contain one or more modifications to the primary amino acid sequence of Smad2 (e.g. SEQ ID NO:2), e.g. mutant "variants" of "wild-type" Smad2. Modifications which create a Smad2 mutant can be made to a Smad2 polypeptide 1) to reduce or eliminate an activity of a Smad2 polypeptide, such as TGF- β receptor mediated phosphorylation; 2) to enhance a property of a Smad2 polypeptide, such as binding to T β R-I or protein stability in an expression system or the stability of protein-protein binding; or 3) to provide a novel activity or property to a Smad2 polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety. Modifications to a Smad2 polypeptide are typically made to the nucleic acid which encodes the Smad2 polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, and the like. Modifications also embrace fusion proteins

comprising all or part of the Smad2 amino acid sequence. Fragments of Smad2 which retain characteristic activities such as the TGF- β receptor binding or interaction activity, the inhibition of wild-type Smad2 activity and the ability to decrease TGF- β -mediated signaling and gene expression. One of ordinary skill in the art can readily determine which fragments retain such activities by preparing fragments of non-wild-type Smad2 using standard procedures such as PCR, restriction endonuclease digestion, or site-directed mutagenesis, and then testing such fragments for non-wild-type Smad2 activities as described herein.

Smad2 polypeptides also include polypeptides referred to herein as "Smad4 binding polypeptides". Such Smad2 polypeptides comprise the portion of Smad 2 which binds to Smad4. As demonstrated below in the examples, Smad4 binding polypeptides can be as small as four amino acids in length, and as large as wild-type Smad2 or larger. It has been discovered that the C-terminus of Smad2 directs the binding of Smad2 to Smad4 and thus preferred examples of Smad4 binding polypeptides include the C-terminal end of Smad2. Examples include the polypeptides of SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5. Other Smad4 binding polypeptides which include SEQ ID NO:3 and additional amino acids of Smad2 C-terminus are contemplated. As will be apparent to one of ordinary skill in the art, Smad4 binding polypeptides also can include non-Smad amino acid sequences.

It has been discovered that the Smad4 binding polypeptide has increased activity when phosphorylated. Thus, it is preferable to prepare and use phosphorylated Smad4 binding polypeptides as exemplified below. One of ordinary skill in the art can prepare such polypeptides by using methods and materials well known in the art.

In general, mutants include pathway-restricted Smad polypeptides such as Smad2 polypeptides which are modified specifically to alter a feature of the polypeptide related to its physiological activity. For example, the experimental evidence provided below demonstrates that serines 465 and 467 are the *in vivo* phosphorylation sites on Smad2. It has also been demonstrated that serine 464 is important for certain Smad2 activities. Thus mutations to one or more of these serine residues can create a Smad2 polypeptide which has different activity than the wild-type Smad2. Such mutants are useful for, *inter alia*, modulation of TGF- β signal transduction *in vitro* and *in vivo*. Smad2 mutants can be used to modulate TGF- β activity for a variety of therapeutic and experimental uses as provided elsewhere herein. Other uses will be apparent to one of ordinary skill in the art.

As demonstrated in the examples below, substitution of alanine residues or aspartic acid

residues for the aforementioned serines in Smad2 results in mutant Smad2 polypeptides with varying degrees of activity with respect to TGF- β receptor interaction, reduction of TGF- β mediated gene expression, reduction of phosphorylation of wild-type Smad2, and the like. It will be clear to one of ordinary skill in the art that other mutations can be made with equal facility using techniques which are standard in the art. Such mutations are not limited to point mutations but also embrace larger deletions and truncations.

In general, variants include Smad2 polypeptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a Smad2 polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present). In addition, mutations which do not result in additional altered activity can be incorporated to enable ease of detection, greater stability, or other properties at the discretion of the artisan.

Mutations of a nucleic acid which encode a pathway-restricted Smad polypeptide such as a Smad2 polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant pathway-restricted Smad polypeptides such as Smad2 polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with the desired properties. Further mutations can be made to variants (or to non-variant Smad2 polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a Smad2 gene or cDNA clone to enhance expression of the polypeptide. The activity of variants of pathway-restricted Smad polypeptides such as Smad2 polypeptides can be tested by cloning the gene encoding the variant Smad polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the variant Smad polypeptide, and testing for a functional capability of the Smad

polypeptides as disclosed herein. For example, the variant Smad2 polypeptide can be tested for inhibition of TGF- β signaling activity as disclosed in the Examples, or for inhibition of Smad2 phosphorylation or for binding to Smad4 as is also disclosed herein. Preparation of other variant polypeptides may favor testing of other activities, such as antibody binding, as will be known to one of ordinary skill in the art.

The skilled artisan will also realize that conservative amino acid substitutions may be made in pathway-restricted Smad polypeptides such as Smad2 polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., the variants retain the functional capabilities of the Smad2 polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the Smad2 polypeptides include conservative amino acid substitutions of SEQ ID NOs:2, 3 and 4. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino-acid substitutions in the amino acid sequence of pathway-restricted Smad polypeptides such as Smad2 polypeptides to produce functionally equivalent variants of Smad2 polypeptides typically are made by alteration of the nucleic acid encoding Smad2 polypeptides (e.g., SEQ ID NOs:2, 3 and 4). Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc. Nat. Acad. Sci. U.S.A.* 82: 488-492, 1985), or by chemical synthesis of a gene encoding a Smad2 polypeptide. Where amino acid substitutions are made to a small unique fragment of a pathway-restricted Smad polypeptide (e.g. a Smad2 polypeptide), such as a Smad4 binding polypeptide, the substitutions can be made by directly synthesizing the peptide. The activity of functionally equivalent fragments of Smad2 polypeptides can be tested by cloning the gene encoding the altered Smad2 polypeptide into a bacterial or mammalian expression vector,

introducing the vector into an appropriate host cell, expressing the altered Smad2 polypeptide, and testing for a functional capability of the Smad2 polypeptides as disclosed herein. Peptides which are chemically synthesized can be tested directly for function, e.g., for binding to Smad4.

Homologs and alleles of the Smad2 nucleic acids of the invention can be identified by conventional techniques. Thus, an aspect of the invention is those nucleic acid sequences which code for Smad2 polypeptides and which hybridize to a nucleic acid molecule consisting of the coding region of SEQ ID NO:1 or SEQ ID NO:14, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 2.5mM NaH₂PO₄(pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulphate; and EDTA is ethylenediaminetetracetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2 x SSC at room temperature and then at 0.1 x SSC/0.1 x SDS at temperatures up to 65°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of Smad2 nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In general homologs and alleles typically will share at least 40% nucleotide identity and/or at least 50% amino acid identity to SEQ ID NO:1 and SEQ ID NO:2 (or SEQ ID NO:14 and SEQ ID NO:15), respectively, in some instances will share at least 50% nucleotide identity and/or at least 65% amino acid identity and in still other instances will share at least 60% nucleotide identity and/or at least 75% amino acid identity. Watson-Crick complements of the foregoing nucleic acids also are embraced by the invention.

In screening for Smad2 nucleic acids, a Southern blot may be performed using the foregoing conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal.

5 The invention also includes degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine residues are encoded by the codons TCA, AGT, TCC, TCG, TCT and AGC. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus,
10 *in vitro* or *in vivo*, to incorporate a serine residue into an elongating Smad2 polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). Other amino acid residues
15 may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of the genetic code.

In one set of embodiments, the nucleic acids of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end
20 of one native nucleotide and the 3' end of another native nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These nucleic acids may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the nucleic acids of the invention also may include
25 "modified" oligonucleotides. That is, the nucleic acids may be modified in a number of ways which do not prevent their transcription or translation but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside
30 linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic

internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamides, carboxymethyl esters and peptides.

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified nucleic acids that encode Smad2 polypeptides, together with pharmaceutically acceptable carriers.

Nucleic acids encoding the pathway-restricted Smad polypeptides such as Smad2 polypeptides of the invention (including TGF- β receptor binding and Smad4 binding polypeptides) may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the nucleic acids in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The compositions should be sterile and contain a therapeutically effective amount of the nucleic acids in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids, phagemids and virus genomes. A cloning vector is one which is able to replicate in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of

plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may
5 be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes
10 which encode enzymes whose activities are detectable by standard assays known in the art (e.g., β -galactosidase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., green fluorescent protein). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

15 As used herein, a coding sequence and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the
20 transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were
25 capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation respectively,
30 such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene. Regulatory sequences may also

include enhancer sequences or upstream activator sequences as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding Smad2 polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell.

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1 α , which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992). The use of the adenovirus as an Adeno.P1A recombinant is disclosed by Warnier et al., in intradermal injection in mice for immunization against P1A (*Int. J. Cancer*, 67:303-310, 1996).

The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

The invention also permits the construction of Smad2 mutant gene "transgenics" in cells and in animals, providing materials for studying certain aspects of TGF- β signal transduction.

The invention as described herein has a number of uses, some of which are described

elsewhere herein. First, the invention permits preparation and isolation of the Smad2 polypeptides with non-wild-type activities such as reduced or even ablated phosphorylation. A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated Smad2 molecules. The polypeptide may be purified from cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce polypeptide. Those skilled in the art also can readily follow known methods for isolating Smad2 polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

The identification of the *in vivo* phosphorylation sites and Smad4 binding interface of pathway-restricted Smad polypeptides such as Smad2 polypeptides also makes it possible for the artisan to diagnose and modulate disorders characterized by aberrant TGF- β signaling activity. These methods involve in certain embodiments contacting the Smad polypeptides of the invention to cells suspected of having elevated TGF- β signaling. The Smad polypeptides of the invention act as dominant-negative inhibitors of TGF- β mediated phosphorylation of wild-type Smad and binding of pathway-restricted Smad polypeptides such as Smad2 polypeptides and Smad4 and thus can modulate elevated TGF- β signaling.

The invention also makes it possible isolate proteins such as T β R-I and Smad4 by the binding of such proteins to pathway-restricted Smad polypeptides such as Smad2 polypeptides as disclosed herein. The identification of this binding also permits one of skill in the art to block the binding of pathway-restricted Smad polypeptides such as Smad2 polypeptides to other proteins, such as T β R-I and Smad4. Binding of the proteins can be effected by introducing into a biological system in which the proteins bind (e.g., a cell) a polypeptide including a Smad T β R-I binding site in an amount sufficient to block the binding. The identification of a Smad4 binding site in Smad2 also enables one of skill in the art to prepare modified proteins, using standard recombinant DNA techniques, which can bind to proteins such as Smad4.

The invention further provides methods for reducing or increasing TGF- β signal transduction in a cell. Such methods are useful *in vitro* for altering the TGF- β signal transduction, for example, in testing compounds for potential to block aberrant TGF- β signal

transduction or increase deficient TGF- β signal transduction. *In vivo*, such methods are useful for modulating growth, e.g., to treat cancer. Increasing TGF- β signal transduction in a cell by, e.g., introducing a dominant negative Smad2 polypeptide in the cell, can be used to provide a model system for testing the effects of putative inhibitors of TGF- β signal transduction. Such methods also are useful in the treatment of conditions which result from excessive or deficient TGF- β signal transduction. TGF- β signal transduction can be measured by a variety of ways known to one of ordinary skill in the art, such as the reporter systems described in the Examples. Various modulators of the activity of pathway-restricted Smad polypeptides such as Smad2 can be screened for effects on TGF- β signal transduction using the methods disclosed herein. The skilled artisan can first determine the modulation of a Smad2 activity, such as TGF- β signaling activity, and then apply such a modulator to a target cell or subject and assess the effect on the target cell or subject. For example, in screening for modulators of Smad2 useful in the treatment of cancer, cells in culture can be contacted with Smad2 modulators and the increase or decrease of growth or focus formation of the cells can be determined according to standard procedures. Smad2 activity modulators can be assessed for their effects on other TGF- β signal transduction downstream effects by similar methods in many cell types.

The invention also provides, as previously noted, "dominant negative" polypeptides derived from Smad2. A dominant negative polypeptide is an inactive variant of a protein, which, by interacting with the cellular machinery, displaces an active protein from its interaction with the cellular machinery or competes with the active protein, thereby reducing the effect of the active protein. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the ligand. Likewise, a dominant negative catalytically-inactive kinase which interacts normally with target proteins but does not phosphorylate the target proteins can reduce phosphorylation of the target proteins in response to a cellular signal. Similarly, a dominant negative transcription factor which binds to a promoter site in the control region of a gene but does not increase gene transcription can reduce the effect of a normal transcription factor by occupying promoter binding sites without increasing transcription.

The end result of the expression of a dominant negative polypeptide in a cell is a reduction in function of active proteins. One of ordinary skill in the art can assess the potential for a dominant negative variant of a protein, and using standard mutagenesis techniques to create one or more dominant negative variant polypeptides. For example, given the teachings contained

herein of mutant Smad2 polypeptides, one of ordinary skill in the art can modify the sequence of the Smad2 polypeptide (SEQ ID NO:2) by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation of the Smad2 cDNA, and the like. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in a selected activity and/or for retention of such an activity (e.g., Smad2 reduction of TGF- β signaling activity). Other similar methods for creating and testing dominant negative variants of a protein will be apparent to one of ordinary skill in the art.

10 Dominant negative pathway-restricted Smad polypeptides (e.g., Smad2 polypeptides) include variants in which the *in vivo* phosphorylation sites are mutated or in which a portion of the Smad4 binding site has been mutated or deleted to reduce or eliminate Smad2 interaction with the Smad4. One of ordinary skill in the art can readily prepare and test Smad2 mutant and variants bearing mutations or deletions for diminution of selected activities.

15 The invention also involves agents such as polypeptides which bind to pathway-restricted Smad polypeptides such as the Smad2 polypeptides of the invention and to complexes of Smad polypeptides and binding partners such as T β R-I or Smad4. Such binding agents can be used, for example, in screening assays to detect the presence or absence of Smad polypeptides and complexes of Smad polypeptides and their binding partners and in purification protocols to
20 isolate Smad polypeptides and complexes of Smad polypeptides and their binding partners. Such agents also can be used to inhibit the native activity of the Smad polypeptides or their binding partners, for example, by binding to such polypeptides, or their binding partners or both.

 The invention, therefore, embraces peptide binding agents which, for example, can be antibodies or fragments of antibodies having the ability to selectively bind to Smad2
25 polypeptides of the invention. Antibodies include polyclonal and monoclonal antibodies, prepared according to conventional methodology (e.g., Kohler and Milstein, *Nature*, 256:495, 1975). As exemplified below, preferred antibodies include antibodies which bind selectively to the phosphorylated tail of Smad2 (Smad2 phospho tail antibodies). Other preferred antibodies include similar phospho tail-specific antibodies for other pathway-restricted Smad proteins (e.g.
30 Smad1, Smad3, Smad5, Smad9). Included in the foregoing are antibodies raised against the four amino acid C-terminal motif of pathway-restricted Smads (SEQ ID NO:5), and antibodies raised against longer phosphorylated fragments of Smad proteins which recognized individual Smad

proteins (e.g. SEQ ID NO:4 and corresponding sequences in other pathway-restricted Smads).

An "antibody" as used herein includes human monoclonal antibodies or functionally active fragments thereof having human constant regions and a Smad protein binding CDR3 region from a mammal of a species other than a human.

5 Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are
10 not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody
15 molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

20 Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated
25 respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining
30 the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. Thus, for example,

PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

5 Humanized monoclonal antibodies may be made by any method known in the art. Humanized monoclonal antibodies, for example, may be constructed by replacing the non-CDR regions of a non-human mammalian antibody with similar regions of human antibodies while retaining the epitopic specificity of the original antibody. For example, non-human CDRs and optionally some of the framework regions may be covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. There are entities in the United States which will
10 synthesize humanized antibodies from specific murine antibody regions commercially, such as Protein Design Labs (Mountain View California).

European Patent Application 0239400, the entire contents of which is hereby incorporated by reference, provides an exemplary teaching of the production and use of
15 humanized monoclonal antibodies in which at least the CDR portion of a murine (or other non-human mammal) antibody is included in the humanized antibody. Briefly, the following methods are useful for constructing a humanized CDR monoclonal antibody including at least a portion of a mouse CDR. A first replicable expression vector including a suitable promoter operably linked to a DNA sequence encoding at least a variable domain of an Ig heavy or light
20 chain and the variable domain comprising framework regions from a human antibody and a CDR region of a murine antibody is prepared. Optionally a second replicable expression vector is prepared which includes a suitable promoter operably linked to a DNA sequence encoding at least the variable domain of a complementary human Ig light or heavy chain respectively. A cell line is then transformed with the vectors. Preferably the cell line is an immortalized mammalian
25 cell line of lymphoid origin, such as a myeloma, hybridoma, trioma, or quadroma cell line, or is a normal lymphoid cell which has been immortalized by transformation with a virus. The transformed cell line is then cultured under conditions known to those of skill in the art to produce the humanized antibody.

As set forth in European Patent Application 0239400 several techniques are well known
30 in the art for creating the particular antibody domains to be inserted into the replicable vector. For example, the DNA sequence encoding the domain may be prepared by oligonucleotide synthesis. Alternatively a synthetic gene lacking the CDR regions in which four framework

regions are fused together with suitable restriction sites at the junctions, such that double stranded synthetic or restricted subcloned CDR cassettes with sticky ends could be ligated at the junctions of the framework regions. Another method involves the preparation of the DNA sequence encoding the variable CDR containing domain by oligonucleotide site-directed mutagenesis. Each of these methods is well known in the art. Therefore, those skilled in the art may construct humanized antibodies containing a murine CDR region without destroying the specificity of the antibody for its epitope.

In preferred embodiments, the humanized antibodies of the invention are human monoclonal antibodies including at least the Smad protein binding CDR3 region of the deposited monoclonal antibody. As noted above, such humanized antibodies may be produced in which some or all of the FR regions of deposited monoclonal antibody have been replaced by homologous human FR regions. In addition, the Fc portions may be replaced so as to produce IgA or IgM as well as human IgG antibodies bearing some or all of the CDRs of the Smad phospho tail antibodies. Of particular importance is the inclusion of the Smad phospho tail protein binding CDR3 region and, to a lesser extent, the other CDRs and portions of the framework regions of the Smad phospho tail antibodies. Such humanized antibodies will have particular clinical utility in that they will specifically recognize Smad proteins, particularly phosphorylated forms, but will not evoke an immune response in humans against the antibody itself.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences.

As used herein the term "functionally active antibody fragment" means a fragment of an antibody molecule including a Smad protein binding region of the invention which retains the Smad binding functionality of an intact antibody having the same specificity as the antibodies disclosed herein. Such fragments are also well known in the art and are regularly employed both

in vitro and *in vivo*. In particular, well-known functionally active antibody fragments include but are not limited to F(ab')₂, Fab, Fv and Fd fragments of antibodies. These fragments which lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)). For example, single-chain antibodies can be constructed in accordance with the methods described in U.S. Patent No. 4,946,778 to Ladner et al. Such single-chain antibodies include the variable regions of the light and heavy chains joined by a flexible linker moiety. Methods for obtaining a single domain antibody ("Fd") which comprises an isolated variable heavy chain single domain, also have been reported (see, for example, Ward et al., *Nature* 341:644-646 (1989), disclosing a method of screening to identify an antibody heavy chain variable region (V_H single domain antibody) with sufficient affinity for its target epitope to bind thereto in isolated form). Methods for making recombinant Fv fragments based on known antibody heavy chain and light chain variable region sequences are known in the art and have been described, e.g., Moore et al., US Patent No. 4,462,334. Other references describing the use and generation of antibody fragments include e.g., Fab fragments (Tijssen, *Practice and Theory of Enzyme Immunoassays* (Elsevier, Amsterdam, 1985)), Fv fragments (Hochman et al., *Biochemistry* 12: 1130 (1973); Sharon et al., *Biochemistry* 15: 1591 (1976); Ehrlich et al., U.S. Patent No. 4,355,023) and portions of antibody molecules (Audilore-Hargreaves, U.S. Patent No. 4,470,925). Thus, those skilled in the art may construct antibody fragments from various portions of intact antibodies without destroying the specificity of the antibodies for the Smad protein epitope.

Functionally active antibody fragments also encompass "humanized antibody fragments." As one skilled in the art will recognize, such fragments could be prepared by traditional enzymatic cleavage of intact humanized antibodies. If, however, intact antibodies are not susceptible to such cleavage, because of the nature of the construction involved, the noted constructions can be prepared with immunoglobulin fragments used as the starting materials; or, if recombinant techniques are used, the DNA sequences, themselves, can be tailored to encode the desired "fragment" which, when expressed, can be combined *in vivo* or *in vitro*, by chemical or biological means, to prepare the final desired intact immunoglobulin fragment.

Thus, the invention in certain aspects involves polypeptides of numerous size and type that bind specifically to pathway-restricted Smad polypeptides (including Smad4 binding polypeptides), and complexes of Smad polypeptides and their binding partners. These

polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries
5 further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention, e.g. those which bind to phosphorylated portions of pathway restricted Smad polypeptides, particularly the C-terminal amino acid motif set forth in SEQ ID NO:5, with or without additional amino acids to confer additional specificity to antibody
10 binding. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to the Smad2 polypeptide, e.g. having one or more phosphorylated serines in the C-terminal tail, or other similarly phosphorylated pathway-restricted Smad
15 polypeptides. This process can be repeated through several cycles of reselection of phage that bind to the Smad polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the Smad polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing
20 part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the Smad polypeptides. Thus, the Smad2 polypeptides of the invention, other phosphorylated pathway-restricted Smads and fragments thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners
25 of the Smad polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of Smad polypeptides and for other purposes that will be apparent to those of ordinary skill in the art.

A Smad2 polypeptide, or a fragment thereof, also can be used to isolate the native binding partners, including, e.g., the TGF- β receptor complex and Smad4. Isolation of such
30 binding partners may be performed according to well-known methods. For example, isolated Smad2 polypeptides can be attached to a substrate (e.g., chromatographic media, such as polystyrene beads, or a filter), and then a solution suspected of containing the TGF- β receptor

complex may be applied to the substrate. If a TGF- β receptor complex which can interact with Smad2 polypeptides is present in the solution, then it will bind to the substrate-bound Smad2 polypeptide. The TGF- β receptor complex then may be isolated. Other proteins which are binding partners for Smad2, such as other Smads or activin receptor complexes, may be isolated
5 by similar methods without undue experimentation.

It will also be recognized that the invention embraces the use of the Smad2 cDNA sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells
10 such as human, mouse, hamster, pig, goat, primate, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. Specific examples include keratinocytes, peripheral blood leukocytes, bone marrow stem cells and embryonic stem cells. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described *supra*, be operably linked to a promoter.

15 When administered, the therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents.

20 The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intracavity, subcutaneous, or transdermal. When antibodies are used therapeutically, a preferred route of administration is by pulmonary aerosol. Techniques for preparing aerosol delivery systems containing antibodies are well
25 known to those of skill in the art. Generally, such systems should utilize components which will not significantly impair the biological properties of the antibodies, such as the paratope binding capacity (see, for example, Sciarra and Cutie, "Aerosols," in Remington's Pharmaceutical Sciences, 18th edition, 1990, pp 1694-1712; incorporated by reference). Those of skill in the art can readily determine the various parameters and conditions for producing antibody aerosols
30 without resort to undue experimentation. When using antisense preparations of the invention, slow intravenous administration is preferred.

Preparations for parenteral administration include sterile aqueous or non-aqueous

solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, 5 Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The preparations of the invention are administered in effective amounts. An effective 10 amount is that amount of a pharmaceutical preparation that alone, or together with further doses, produces the desired response. In the case of treating cancer, the desired response is inhibiting the progression of the cancer. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. This can be monitored by routine methods or can be monitored according to 15 diagnostic methods of the invention discussed herein.

The invention also contemplates gene therapy. The procedure for performing *ex vivo* gene therapy is outlined in U.S. Patent 5,399,346 and in exhibits submitted in the file history of that patent, all of which are publicly available documents. In general, it involves introduction *in vitro* of a functional copy of a gene into a cell(s) of a subject which contains a defective copy of 20 the gene, and returning the genetically engineered cell(s) to the subject. The functional copy of the gene is under operable control of regulatory elements which permit expression of the gene in the genetically engineered cell(s). Numerous transfection and transduction techniques as well as appropriate expression vectors are well known to those of ordinary skill in the art, some of which are described in PCT application WO95/00654. *In vivo* gene therapy using vectors such as 25 adenovirus, retroviruses, herpes virus, and targeted liposomes also is contemplated according to the invention.

The invention further provides efficient methods of identifying pharmacological agents or lead compounds for agents active at the level of a cellular function modulatable by apathway-restricted Smad polypeptide or fragment such as a Smad2 polypeptide or fragment thereof. In 30 particular, such functions include TGF- β superfamily signal transduction and formation of TGF- β superfamily receptor-Smad2 and Smad2-Smad4 protein complexes. Generally, the screening methods involve assaying for compounds which interfere with a Smad activity such as TGF- β

superfamily receptor-Smad binding, etc. Such methods are adaptable to automated, high throughput screening of compounds. The target therapeutic indications for pharmacological agents detected by the screening methods are limited only in that the target cellular function be subject to modulation by alteration of the formation of a complex comprising a pathway-restricted Smad polypeptide such as a Smad2 polypeptide of the invention or fragment thereof and one or more natural Smad intracellular binding targets, such as a TGF- β superfamily receptor. Target indications include cellular processes modulated by TGF- β , BMP and/or activin signal transduction following receptor-ligand binding.

A wide variety of assays for pharmacological agents are provided, including, labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays, cell-based assays such as two- or three-hybrid screens, expression assays, etc. For example, three-hybrid screens are used to rapidly examine the effect of transfected nucleic acids on the intracellular binding of pathway-restricted Smad or Smad fragments to specific intracellular targets. The transfected nucleic acids can encode, for example, combinatorial peptide libraries or antisense molecules. Convenient reagents for such assays, e.g., GAL4 fusion proteins, are known in the art. An exemplary cell-based assay involves transfecting a cell with a nucleic acid encoding a Smad2 polypeptide fused to a GAL4 DNA binding domain and a nucleic acid encoding a Smad4 domain which interacts with Smad2 fused to a transcription activation domain such as VP16. The cell also contains a reporter gene operably linked to a gene expression regulatory region, such as one or more GAL4 binding sites. Activation of reporter gene transcription occurs when the Smad2 and Smad4 fusion polypeptides bind such that the GAL4 DNA binding domain and the VP16 transcriptional activation domain are brought into proximity to enable transcription of the reporter gene. Agents which modulate a Smad2 polypeptide mediated cell function are then detected through a change in the expression of reporter gene. Methods for determining changes in the expression of a reporter gene are known in the art.

Pathway-restricted Smad fragments used in the methods, when not produced by a transfected nucleic acid are added to an assay mixture as an isolated polypeptide. Pathway-restricted Smad polypeptides such as Smad2 polypeptides preferably are produced recombinantly, although such polypeptides may be isolated from biological extracts. Recombinantly produced Smad polypeptides include chimeric proteins comprising a fusion of a Smad protein with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, sequence specific nucleic acid binding (such as GAL4), enhancing

stability of the Smad polypeptide under assay conditions, or providing a detectable moiety, such as green fluorescent protein or Flag epitope as provided in the examples below.

The assay mixture is comprised of a natural intracellular Smad binding target such as a TGF- β superfamily receptor or fragment thereof capable of interacting with a Smad polypeptide.

5 While natural Smad binding targets may be used, it is frequently preferred to use portions (e.g., peptides or nucleic acid fragments) or analogs (i.e., agents which mimic the Smad binding properties of the natural binding target for purposes of the assay) of the Smad binding target so long as the portion or analog provides binding affinity and avidity to the Smad fragment measurable in the assay.

10 The assay mixture also comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate agents encompass numerous chemical classes, although
15 typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500, preferably less than about 1000 and, more preferably, less than about 500.

Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides and/or nucleic acids, and typically include at least an amine, carbonyl, hydroxyl or
20 carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate agents also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or
25 structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified nucleic acids as defined herein are also contemplated.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and
30 directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds

in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily be modified through conventional chemical, physical, and biochemical means. Further, known pharmacological agents may be subjected to directed or random chemical modifications such as
5 acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the agents.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also
10 reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease, inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the Smad2 polypeptide specifically
15 binds the cellular binding target, a portion thereof or analog thereof. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high
20 throughput screening, and typically are between 0.1 and 10 hours.

After incubation, the presence or absence of specific binding between the Smad2 polypeptide and one or more binding targets is detected by any convenient method available to the user. For cell free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways.
25 Conveniently, at least one of the components is immobilized on a solid substrate, from which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximum signal to noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

30 Separation may be effected for example, by removing a bead or dipstick from a reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead, particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably

includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in specific bindings such as salts, buffer, detergent, non-specific protein, etc. Where the solid substrate is a magnetic bead, the
5 beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as two- or three-hybrid screens. The transcript resulting from a reporter gene transcription assay of Smad2 polypeptide interacting with a target molecule typically encodes a directly or indirectly detectable product, e.g., β -galactosidase activity, luciferase activity, and the like. For cell free
10 binding assays, one of the components usually comprises, or is coupled to, a detectable label. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc.) or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horseshoe peroxidase, etc.). The label may be bound to a Smad binding partner, or incorporated into the structure of the binding partner.

15 A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, etc. Methods for
20 detecting the labels are well known in the art.

The invention provides Smad-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, Smad2-specific pharmacological agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper
25 utilization of a pathway involving Smad2, e.g., TGF- β induced phosphorylation of Smad2, Smad4-Smad2 complex formation, etc. Novel Smad-specific binding agents include pathway restricted Smad-specific antibodies (e.g. phospho tail antibodies) and other natural intracellular binding agents identified with assays such as two hybrid screens, and non-natural intracellular binding agents identified in screens of chemical libraries and the like.

30 In general, the specificity of Smad2 binding to a binding agent is shown by binding equilibrium constants. Targets which are capable of selectively binding a Smad polypeptide preferably have binding equilibrium constants of at least about 10^7 M^{-1} , more preferably at least

about 10^8 M^{-1} , and most preferably at least about 10^9 M^{-1} . The wide variety of cell based and cell free assays may be used to demonstrate pathway-restricted Smad-specific binding. Cell based assays include one, two and three hybrid screens, assays in which Smad-mediated transcription is inhibited or increased, etc. Cell free assays include Smad-protein binding assays, immunoassays, etc. Other assays useful for screening agents which bind pathway-restricted Smad polypeptides such as Smad2 polypeptides include fluorescence resonance energy transfer (FRET), and electrophoretic mobility shift analysis (EMSA).

Various techniques may be employed for introducing nucleic acids of the invention into cells, depending on whether the nucleic acids are introduced *in vitro* or *in vivo* in a host. Such techniques include transfection of nucleic acid- CaPO_4 precipitates, transfection of nucleic acids associated with DEAE, transfection with a retrovirus including the nucleic acid of interest, liposome mediated transfection, and the like. For certain uses, it is preferred to target the nucleic acid to particular cells. In such instances, a vehicle used for delivering a nucleic acid of the invention into a cell (e.g., a retrovirus, or other virus; a liposome) can have a targeting molecule attached thereto. For example, a molecule such as an antibody specific for a surface membrane protein on the target cell or a ligand for a receptor on the target cell can be bound to or incorporated within the nucleic acid delivery vehicle. For example, where liposomes are employed to deliver the nucleic acids of the invention, proteins which bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake. Such proteins include capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like. Polymeric delivery systems also have been used successfully to deliver nucleic acids into cells, as is known by those skilled in the art. Such systems even permit oral delivery of nucleic acids.

EXAMPLES

Materials and Methods

30 *Cell lines*

COS-1 cells and mink lung epithelial (Mv1Lu) cells were obtained from American Type Culture Collection. Cells were cultured in DMEM (GIBCO-BRL) with 10% fetal bovine serum,

100 units/ml of penicillin and 50 µg/ml of streptomycin.

Constructs and cell transfection

Expression of plasmids for TβR-I, TβR-II and Smad2 were previously described (ten
5 Dijke et al., *Science*, 264:101-104, 1994; Nakao et al., 1996). Smad2 mutants were made by a
polymerase chain reaction (PCR)-directed approach and subcloned in pcDNA3 vector.
Mutations and sequences of the exchanged restriction fragments in Smad2 cDNA were
confirmed by DNA sequencing. Transient transfections were performed using a DEAE-dextran
protocol, as described (Cárcamo et al., *Mol. Cell. Biol.*, 14:3810-3821, 1994).

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[³²P]orthophosphate labeling, tryptic phosphopeptide mapping, two-dimensional phosphoamino acid analysis and automated Edman degradation

[³²P]orthophosphate labeling, tryptic phosphopeptide mapping, two-dimensional
phosphoamino acid analysis and automated Edman degradation were performed as previously
15 described (Souchelnytskyi et al., *EMBO J.*, 15:6231-6240, 1996). In brief, subconfluent cells
were labeled in phosphate-free medium containing 0.5% dialyzed FCS, 20 mM HEPES, pH 7.2,
and [³²P]orthophosphate (1.0 mCi/ml). After stimulation with TGF-βI for 60 min, the cells were
washed and lysed in lysis buffer (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.5% Triton X-100, 5
mM NaF, 10 mM Na₄P₂O₇, 1 mM Na₃VO₄, 1 mM phenylmethylsulphonyl fluoride (PMSF), 100
20 U/ml aprotinin). The cell lysates were subjected to immunoprecipitation using the SED antisera
against Smad2 (SED; Nakao et al., 1996); immunoprecipitates were then subjected to SDS-
PAGE and transferred to a nitrocellulose membrane. The phosphorylated Smad2 proteins were
excised from the filter and digested *in situ* with trypsin (modified sequencing grade; Promega).
Two-dimensional phosphopeptide mapping was done using the Hunter thin-layer electrophoresis
25 (HTLE-7000; CBS Scientific), essentially as described by Boyle et al. (*Methods Enzymol.*,
201:110-149, 1991). First dimension electrophoresis was performed in pH 1.9 buffer (formic
acid/glacial acid/water; 44:156:1800; v/v/v) for 27 min at 2000 V, and chromatography in the
second dimension in isobutyric acid/n-butanol/pyridine/glacial acetic acid/water (1250:38:96: 58:
558; v/v/v/v/v). After exposure, phosphopeptides were eluted from the plates in pH 1.9 buffer
30 and lyophilized; aliquots of the samples were then subjected to two-dimensional phosphoamino
acid analysis and automated Edman degradation in parallel. For radiochemical sequencing, the
eluted phosphopeptides were coupled to Sequelon-AA membrane (Millipore) by use of

carbodiimide coupling, according to standard procedures as described by the manufacturer, and Edman degradation was performed using an Applied Biosystems sequencer (Model 477A). Released phenylthiohydantoin amino acid-derivatives from each cycle were spotted onto thin-layer chromatography plates. The radioactivity in each spot was quantitated by exposure on a FujiX Bio-Imager.

Iodination of TGF- β 1 and affinity cross-linking

TGF- β 1 was iodinated using the chloramine T method according to Frolik et al. (*J. Biol. Chem.*, 259:10995-11000, 1984). Cross-linking was performed as previously described (Franzén et al., *Cell*, 75:681-692, 1993). Complexes of Smad2 and TGF- β receptors, cross-linked with 125 I-TGF- β , were immunoprecipitated with Smad2 antiserum (SED; Nakao et al., 1996) and subjected to SDS-PAGE. Gels were dried and exposed on a FujiX Bio-Imager. To determine equality of T β R's expression, aliquots of cell lysates were subjected to immunoprecipitation using anti-T β R-I antibodies, and analyzed by SDS-PAGE and autoradiography as previously described (Franzén et al., 1993). To determine the expression of Smad proteins, cell lysates were separated by SDS-PAGE, electrotransferred to nitrocellulose membrane, immunoblotted with the Smad2 antiserum and developed using an enhanced chemiluminescence detection system (Amersham).

Transcriptional response assay

MvLu cells were transiently transfected with p3TP-Lux (Cárcamo et al., *Mol. Cell. Biol.*, 15:1573-1581, 1995) in the absence or presence of Smad2 expression plasmids by using the DEAE-dextran method. After transfection cells were incubated for 24 h in DMEM with 10% FBS, and then incubated with 0.1% FBS for 5 h, after which TGF- β 1 was added. Luciferase activity in the cell lysate was measured after 22-24 h using the luciferase assay system (Promega Biotech), according to the manufacturer's protocol using an LKB Luminometer (LKB Bromma).

Peptide synthesis and coupling to solid support

The peptides KKKSSMS (SEQ ID NO:6) and KKKYTQMGSPPVRCSSMS (SEQ ID NO:7) were synthesized using an Applied Biosystems peptide synthesizer (model ABI 430A) by Fmoc chemistry. The corresponding peptides with the two most C-terminal serine residues phosphorylated were synthesized using phosphorylated F-moc serine residue derivatives during

synthesis. The peptides were analyzed by plasma desorption mass spectrometry using an Applied Biosystems Bio Ion 20 instrument. Peptide fractions were freeze-dried and stored under dry conditions. Peptides were coupled to activated CNBr Sepharose 4B (Pharmacia) through primary amino groups according to the manufacturer's protocol; the three lysines at the N-terminus were included to facilitate efficient coupling. The efficiency of coupling was determined by measuring the OD₂₈₀ (or OD₂₁₅ for short peptides) of the peptide solution before and after coupling. The coupling efficiencies of all four peptides were nearly 100%.

Preparation of GST-fusion proteins

cDNA for Smads were cloned into pGEX vectors (Pharmacia LKB), and fusion proteins were prepared and absorbed essentially as described (Smith and Johnson, *Gene*, 63:31-40, 1988).

Association of phosphorylated and nonphosphorylated Smad2 peptides with GST fusion proteins

Phosphorylated and nonphosphorylated Smad2-derived C-terminal peptides were incubated with GST-Smad fusion proteins in phosphate buffered saline containing 1% Triton (PBS-T) with 0.1% BSA overnight at 4°C. After washing five times with PBS-T with 0.1% BSA and twice with PBS-T, the samples were subjected to SDS-PAGE. Proteins were electrotransferred to nitrocellulose membrane and immunoblotted with GST antiserum (gift from Aino Ruusala) and developed using an enhanced chemiluminescence detection system (Amersham).

Example 1: Mapping of in vivo phosphorylation sites in Smad2

TGF- β receptor activation leads to phosphorylation of Smad2 on serine and threonine residues (Eppert et al., 1996; Macías-Silva et al., 1996; Nakao et al., 1996). In order to localize the phosphorylated residues in Smad2, two-dimensional tryptic phosphopeptide mapping was performed. Untransfected Mv1Lu cells, Mv1Lu cells transiently transfected with Smad2 alone, and COS-1 cells transiently transfected with Smad2 together with T β R-I and T β R-II, were labeled with [³²P]orthophosphate and incubated with or without TGF- β 1. Thereafter, cell lysates were subjected to immunoprecipitation using a Smad2 antiserum, followed by SDS-PAGE and autoradiography. Plates were exposed and analyzed by using a FujiX Bio-Imager. Sample

application points are shown by small black squares. In the absence of TGF- β 1, Smad2 was only phosphorylated at low stoichiometry; phosphorylation was dramatically enhanced after ligand-stimulation (Fig. 1A).

The Smad2 phosphoproteins of the experiment shown in Fig. 1A were transferred to a membrane, cut out (from lanes 3 (B), 4 (C), 5 (E) and 6 (F)) and subjected to tryptic digestion. After digestion with trypsin, peptides were resolved by high-voltage electrophoresis and thin-layer chromatography. Analysis of the two-dimensional maps of ^{32}P -labeled phosphopeptides revealed about 16 spots of different intensities (Fig. 1B, C, D). In the absence of ligand-stimulation, a broad smear (spot 18) and few faint spots were seen. The abundantly phosphorylated peptide (spot 18) was immunoprecipitated with antiserum raised against a peptide from a sequence in a large tryptic peptide that covers almost completely the proline-rich linker sequence between the MH1 and MH2 domains. This phosphopeptide contained phosphoserine as well as phosphothreonine.

Stimulation by TGF- β 1 led to the appearance and induction of multiple spots; in particular, seven highly negatively charged phosphopeptides were seen (spots 5, 6, 6n, 12a, 12b, 12c and 15). Phosphopeptide maps of endogenous and overexpressed Smad2 in Mv1Lu cells were identical, albeit signal intensity was higher on maps of overexpressed Smad2. Overexpression of Smad2 with T β R-I and T β R-II in COS-1 cells and incubation with ligand also led to stimulation of Smad2 phosphorylation (Fig. 1A). Tryptic phosphopeptide maps of Smad2 phosphorylated *in vivo* in these cells revealed a nearly identical pattern as compared to phosphorylated Smad2 from Mv1Lu cells (Fig. 1E, F, G). Phosphoamino acid analysis of spots 1 through 18, revealed that all were phosphorylated on serine residues except for spot 11 and 18 that contained both phosphoserine and phosphothreonine residues (data not shown). None of the spots contained phosphotyrosine.

The phosphopeptide corresponding to spot 15 in Fig. 1F was subjected to phosphoamino acid analysis. The migration of phosphorylated serine (Sp), threonine (Tp) and tyrosine (Yp), used as standards, is shown. To identify the positions of phosphorylated residues in the phosphopeptides, the release of radioactivity upon Edman degradation of peptides extracted from the two-dimensional chromatography plate was determined. The elution positions of ^{32}P -labeled amino acids are shown and aligned to the sequence of the single possible Smad2-derived tryptic peptide containing serine residues in the third and fifth position. The peptide corresponding to spot 15, which contained only phosphoserine, yielded ^{32}P -radioactivity release in the third and

fifth cycles, with some trailing in the following cycles which is characteristic for this method. This indicates that Ser465 and Ser467 in the C-terminus of Smad2 were phosphorylated, since the corresponding tryptic peptide is the only one in the Smad2 with serine residues in position three and five (Fig. 2A, B). The carboxy-terminal tryptic peptide also contained another phosphorylatable amino acid, i.e., Ser464. However, radiochemical sequencing and phosphoamino acid analysis did not reveal phosphoserine at position two in phosphopeptides with the migration position expected if Ser464 would have been phosphorylated alone or in combinations with Ser465 and Ser467. Thus, Ser464 in Smad2 appears not to be phosphorylated in response to TGF- β -stimulation.

10 In order to confirm that Ser465 and Ser467 are phosphorylation sites in Smad2, these residues and the neighboring Ser464 were mutated to alanine residues singly and in combinations. Wild-type Smad2 (W.T.), as well as Smad2/S464A (S464A), Smad2/S465,467A (S465,467A) and Smad2/S464,465,467A (S464,465,467A) mutants were subjected to two-dimensional phosphopeptide mapping. COS-1 cells were transiently transfected with Smad2 or
15 Smad2 mutants together with T β R-I and T β R-II, and were labeled with [32 P]orthophosphate. After treatment with TGF- β , wild-type Smad2 or Smad2 mutants were immunoprecipitated and subjected to two-dimensional tryptic phosphopeptide mapping. Sample application points are shown by black squares. The arrows show the migration position of the Ser465 and Ser467 containing tryptic peptide. Analysis of two-dimensional tryptic phosphopeptide maps of wild-
20 type and mutant Smad2 from 32 P-labeled TGF- β -stimulated COS-1 cells, revealed that spot 15, corresponding to a peptide with Ser465 and Ser467 phosphorylated, was not seen in the phosphopeptide map of the Smad2/S465A mutant contained spot 15; the map of the Smad2/S464A mutant was in fact identical to that of wild-type Smad2. Moreover, the phosphopeptide map of the triple Smad2/S464, 465, 467A mutant also lacked spot 15, and was
25 identical to that of the Smad2/S465, 467A mutant. Thus, Ser465 and Ser467 are *in vivo* phosphorylation sites in Smad2.

Fig. 2D shows a schematic illustration of Smad2 and C-terminal sequences of Smad1, Smad2, Smad3 and Smad5. Conserved residues are boxed. The C-terminal motif SS(M/V)S (SEQ ID NO: 5) is found in Smad1, Smad2, Smad3, Smad5 and Smad9, and therefore, it is
30 possible that all these Smads are direct substrates for their appropriate activated receptors. In accordance with this possibility a phosphopeptide map of Smad3 from TGF- β -stimulated cells revealed phosphopeptides with similar migration as spot 15 of Smad2; no such spot was found in

a map of Smad4, which lacks the C-terminal SS(M/V)S motif.

Example 2: *Phosphorylation of Ser465 requires that Ser467 is phosphorylated*

Since Ser465 and Ser467 are located in close proximity of each other, the possibility that they are phosphorylated sequentially was examined. TBR-I-mediated phosphorylation of Smad2 mutants in which Ser465 or Ser467 were converted to alanine or aspartic acid residues was characterized in transfected COS-1 cells. Two-dimensional tryptic phosphopeptide mapping of wild-type Smad2 and Smad2/S465A, Smad2/S465D, Smad2/S467A and Smad2/S467D mutants after co-expression with TGF- β receptors and stimulation with TGF- β , performed as described above, showed loss of spot 15 in all cases. The arrows indicate the migration position of the C-terminal tryptic peptides, which is absent on the map of the Smad2/S467A mutant. Notably, a new spot appeared in the maps of the Smad2/S465A, Smad2/S465D and Smad2/S467D mutants, but not in the map of the Smad2/S467A mutant (Fig. 3A). The new peptide had a shorter migration distance in electrophoresis at pH 1.9 compared to that of spot 15, which is in agreement with a lower degree of phosphorylation of the novel phosphopeptide and suggested that it was phosphorylated at one rather than two residues. Edman degradation elution profiles of the new phosphopeptides appeared on maps of the Smad2/S465A and Smad2/S467D mutants are shown in Fig. 3B. The ^{32}P -radioactivity released in each cycle was measured. The amino acid sequences of the C-terminals of the Smad2 mutants are presented along with the fraction numbers. When radiochemical sequencing of the novel spot from the map of the Smad2/S465A mutant was performed, radioactivity eluted at the fifth cycle, which is consistent with the expected phosphorylation of the C-terminal tryptic peptide at Ser467 after mutation of Ser465. The fact that the new spot was not seen in the map of the Smad2/S467A mutant suggests that phosphorylation of Ser465 requires phosphorylation of Ser467. The finding that the map of a Smad2/S467D mutant showed the novel spot, suggested that introduction of a negative charge at position 467 could rescue the phosphorylation of Ser465. Radiochemical sequencing of the spot revealed that the peak of radioactivity eluted at the third cycle. This result indicates that Ser465 was phosphorylated in the C-terminal peptide of the Smad2/S467D mutant, and suggests that the requirement for phosphorylation at Ser467 was bypassed by the introduction of a negative charge at this residue.

Example 3: *Mutation of Ser464, Ser465 and/or Ser467 in Smad2 interferes with TGF- β -*

mediated signaling

To reveal the importance of Ser464, Ser465 and Ser467 for TGF- β signaling, the ability of Smad2 mutants, in which these residues were replaced with alanine or aspartic acid residues singly or in combinations, to block the TGF- β -mediated transcriptional response was measured using a p3TP-Lux reporter plasmid (Fig. 4). Mv1Lu cells were transiently transfected with p3TP-Lux plasmid alone or in the presence of Smad2 or Smad2 mutant expression plasmids. Luciferase activity was determined before (■) or after (□) stimulation with 10 ng/ml of TGF- β 1. The values were normalized for transfection efficiency using the β -gal reporter gene under transcriptional control of cytomegalovirus promoter. Representative results of three independent experiments are shown. Consistent with previous observations (Zhang et al., 1996), no difference in signaling in the absence or presence of transfected wild-type Smad2 was found, indicating that level of endogenous Smad2 is sufficient for full response. Expression of any Smad2 with Ser465 or Ser467 replaced with alanine residues(s) led to a decrease of the luciferase signal. Introduction of aspartic acid residues, in order to mimic the negative charge of the phosphate group, did not rescue the stimulation of luciferase expression. Notably, Smad2/Ser464A also acted as a dominant negative inhibitor. However, in some experiments, the inhibitory effect of this mutant on TGF- β signaling was less pronounced than those of Smad2/S465A and Smad2/S467A mutants. Thus, Ser464 and in particular the phosphorylatable residues Ser465 and Ser467 in Smad2, are required for TGF- β -induced p3TP-Lux transcriptional response.

Example 4: Association of wild-type Smad2 and Smad2 mutants with T β R-I

The interaction of Smad2 with the TGF- β receptor complex was investigated by co-expression of wild-type Smad2 and Smad2 mutants with TGF- β receptor in COS-1 cells (Fig. 5). COS-1 cells were transfected with wild-type Smad2 (W.T.) or Smad2 mutants in combination with T β R-II and wild-type (W.T.) or kinase-inactive (K.R.) forms of T β R-I. Constructs for WT and KR T β R-I tagged at C-terminus with HA epitope were used. The receptors were affinity cross-linked with 125 I-TGF- β 1. Cell lysates were subjected to immunoprecipitation with Smad2 antiserum and analyzed by SDS-PAGE and autoradiography. Migration position of T β R-I and T β R-II are shown. Smad2-receptor interaction was determined by the ability of an antiserum against Smad2 to co-immunoprecipitate the receptors, cross-linked with 125 I-TGF- β 1. In accordance with previous results (Macias-Silva et al., 1996), it was

determined that wild-type Smad2 interacted with T β R-I, provided that T β R-I was kinase-inactive and phosphorylated by T β R-II kinase; in contrast, a Smad2 mutant with the three C-terminal serines altered to alanines, was able to bind with high affinity also to activated wild-type T β R-I. The single and double serine mutants of Smad2 were analyzed in a similar experimental setup for their abilities to interact with wild-type T β R-I or a kinase-inactive T β R-I mutant in complex with T β R-II. The double mutant interacted with T β R-I in a similar manner as the triple mutant. In addition, the single mutants were also found to interact with wild-type T β R-I. Interestingly, although Ser464 is not an *in vivo* phosphorylation site, Smad2/S464A interacted with T β R-I as efficiently as the Smad2/S465A and Smad2/S467A mutants (Fig. 5). T β R-I and T β R-II were expressed at equal levels, as confirmed by immunoprecipitation of the crosslinked ¹²⁵I-TGF- β -receptor complex, followed by SDS-PAGE and autoradiography; also Smad2 was expressed at equal levels as determined by immunoblotting of cell lysates, resolved by SDS-PAGE, with Smad2 antiserum (data not shown). These data are in concordance with the dominant negative effects of Smad2 and mutated at Ser464, Ser465 and/or Ser467 on TGF- β -mediated stimulation of gene expression (Fig. 4), and suggests that the mechanism involves competition of the Smad2 mutants and endogenous Smad2 for binding to T β R-I.

Example 5: GST-Smad4 binds with higher affinity to phosphorylated peptides derived from the C-terminus of Smad2 than to the corresponding non-phosphorylated peptides

To investigate whether phosphorylated Ser465 and Ser467 of Smad2 are directly involved in heteromeric interaction with Smad4, phosphorylated and non-phosphorylated peptides corresponding to the C-terminus of Smad2 were made and tested for their ability to bind GST-Smad4 fusion proteins. Short C-terminal peptide of Smad2 SSMS (SNp; SEQ ID NO: 3) or its phosphorylated counterpart (SPp), as well as long C-terminal-peptide TQMGSPSVRCSSMS (LNp; SEQ ID NO: 4) or its phosphorylated counterpart (LPp), coupled to CNBr-Sepharose (peptide beads), were incubated with GST or GST-Smad4 fusion protein. For competition large excesses (200 μ M) of non-phosphorylated short (SNp) or long (LNp) peptides or their phosphorylated counterpart (SPp and LPp, respectively) were used (block). Proteins bound to the beads, were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-GST antibodies. Migration positions of GST and GST-Smad4 are shown by double arrows. Molecular masses of standard proteins are indicated on the left side on gel in which protein-stained GST and GST-Smad4 are shown. It was found that

Smad4 bound to a doubly phosphorylated peptide containing the four C-terminal amino acid residues of Smad2 (SSpMSP SEQ ID NO: 3), but weakly (or not) to the corresponding non-phosphorylated counterpart (SSMS) (Fig. 6). We also found that the phosphorylated form of a longer peptide containing the 14 C-terminal amino acid residues of Smad2

- 5 (TQMGSPSVRCSSpMSP SEQ ID NO: 4) bound GST-Smad4 even more efficiently than the SSpMSP peptide. The phosphorylated long peptide also bound GST-Smad4 more efficiently, than the non-phosphorylated counterpart, however, a considerable binding was observed also to the long non-phosphorylated peptide. This indicates that interaction between Smad2 and Smad4 is not only dependent on phosphorylation of Ser465 and Ser467 in Smad2, but involves also
10 interaction with the sequence upstream of the phosphorylation sites. The interaction was shown to be specific, since it was blocked by an excess of short or long phosphorylated peptides, but not by an excess of an irrelevant phosphopeptide, phosphoserine, or peptides derived from Smad2; the GST protein showed no binding to phosphorylated peptides (Fig. 6; and data not shown).

15 **Example 6: Preparation of Smad2 phospho tail antibody.**

- Rabbit antisera to TGF- β -receptor-mediated phosphorylated Smad2 was made against the phosphorylated tail of Smad2. A three lysine peptide (KKK) was added to the N-terminus of the amino acids of SEQ ID NO:3 to facilitate coupling to carrier protein. The resulting peptide, KKKSSMS (SEQ ID NO:6), in which underlined serines (amino acids 5 and 7, equivalent to
20 Ser465 and Ser467 of Smad2) are phosphoserines, was coupled to keyhole limpet hemocyanin with glutaraldehyde, mixed with Freund's adjuvant, and used to immunize rabbits according to standard protocols for preparation of polyclonal antibodies. Blood was drawn from immunized rabbits and antisera prepared according to standard procedures. Characterization of the phosphoserine specific antisera (referred to as Smad2 phospho tail antibody) is described in the
25 following examples. Additional Smad2 phosphoserine specific antisera are prepared as described above, using longer Smad2 peptides such as a 14mer peptide (SEQ ID NO:4), 8mer peptides corresponding to amino acids 4-11, 5-12, 6-13 and 7-14 of SEQ ID NO:4, 4mer peptides corresponding to amino acids 8-11, 9-12, and 10-13 of SEQ ID NO:4. Other peptides can be chosen as desired to prepare still other Smad2 phosphoserine specific antisera. One or
30 more of the serine residues in the foregoing peptides are phosphorylated.

Example 7: Smad2 phospho tail antibody recognizes TGF- β type I receptor mediated

phosphorylated Smad2 by Western blot analysis.

COS cells were transfected with TGF- β type I and type II receptors and Smad2 expression plasmids in the absence or presence of different amounts of Smad7 expression plasmid. Cells were treated without or with TGF- β , and cell lysates were prepared and used for Western blotting using the Smad2 phospho tail antibody prepared in Example 6. The results of these experiments are shown in Fig. 7. In the absence of Smad7 and TGF- β , Smad2 is weakly phosphorylated (lane 1), which is increased after ligand addition (lane 4). The phosphorylation in the absence of ligand is caused by ligand independent heteromeric complex formation of receptors in COS cells. In the presence of increasing amounts of Smad7, which inhibits receptor dependent Smad2 phosphorylation on Ser465 and Ser467 in C-tail, a dose dependent decrease in Smad2 phosphorylation was observed. Thus the Smad2 phospho tail antibody recognized TGF- β type I receptor mediated phosphorylated Smad2. The same blot was reprobed with Smad2 specific antibody; the equal expression of Smad2 in each transfection was shown.

Example 8: Smad2 phospho tail antibody recognizes phosphorylated Smad2 in extracts from metabolically labeled and TGF- β stimulated cells.

To determine whether the Smad2 phospho tail antibody binds Smad2 phosphorylated following TGF- β stimulation, the glioblastoma cell line U-343 MGa 35L was metabolically labeled with ^{35}S -methionine and ^{35}S -cysteine for 4 hours. During the last hour of labeling cells were incubated with or without 10 ng/ml TGF- β 1. Cell lysates were precleaned by incubation with protein A-Sepharose beads. After centrifugation to remove the protein A-Sepharose beads, the cell extracts were subjected to immunoprecipitation using Smad-specific antisera. Antiserum '543' recognizes Smad2, '542' is specific for Smad3, 'DPC' is raised towards Smad 4 and '597' is the Smad2 phospho tail antibody prepared in Example 6. To show specificity of the antisera, controls were included containing the respective peptides to which the antisera were raised. Incubations were done for 2 hours after which the immunocomplexes were collected on protein-A Sepharose beads. The beads were washed three times with lysis buffer (125 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM PMSF, 1.5% trasylol, and 1% Triton X-100), three times with RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 0.5% Triton X-100, 50 mM Tris-HCl pH 8.0), and three times with high salt buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1% Triton X-100). Immunocomplexes were separated on a 8.5% SDS-PAGE gel and visualized using a Fuji-X BioImager. As shown in Fig. 8, U-343 MGa 35L clearly expressed

Smad2 protein as specifically recognized by the 543 antiserum (lanes 1, 2, 9 and 10), while Smad4 was brought down by the DPC antibody (lanes 5, 6, 13, 14). Smad3 protein was not detected in these cells. In the absence of TGF- β 1, the Smad2 phospho tail antiserum (597) did not detect any Smad protein (lanes 7, 8). Upon treatment of the cells for one hour with 10 ng/ml of TGF- β 1, Smad2 phospho tail antibody clearly and specifically recognized phosphorylated Smad2 (lanes 11, 12). These data indicate that the Smad2 phospho tail antiserum can be successfully applied for the detection of TGF- β induced phosphorylated Smad2 present in extracts from metabolically labeled cells.

10 **Example 9: Preparation of phospho tail antibodies which recognize pathway restricted Smad1, Smad3, Smad5, and Smad9.**

Rabbit antisera to TGF- β -receptor-mediated phosphorylated Smad1, Smad3, Smad5, and Smad9 are made against peptides representing the phosphorylated tail of these Smad proteins. A three lysine peptide (KKK) is added to the N-terminus of the amino acids of SEQ ID NO:8 (SSVS) to facilitate coupling to carrier protein. The resulting peptides, e.g. KKKSSVS (SEQ ID NO:9), in which one or more of the serines are phosphoserines is coupled to keyhole limpet hemocyanin with glutaraldehyde. Longer peptides are used as needed to prepare Smad-specific antibodies. For example, additional peptides are prepared by the addition of the KKK linker to longer peptides derived from the C-terminus of Smad1, Smad3, Smad5 and Smad9, e.g. 14mers analogous to SEQ ID NO:4 including SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:13, or intermediate length peptides such as 8mers corresponding to amino acids 7-14 of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:13. Still other peptides are used to prepare antibodies to pathway-restricted Smad proteins, including Smad C-terminal peptides which lack the two most C-terminal amino acids (e.g., 4mers corresponding to amino acids 9-12 of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:13, or 8mers corresponding to amino acids 5-12 of SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:13). For all of the foregoing peptides, one or more of the C-terminal serine residues are phosphorylated.

The coupled peptides are mixed with Freund's adjuvant and is used to immunize rabbits according to standard methods for preparing polyclonal antibodies. Blood is drawn from immunized rabbits and antisera prepared according to standard procedures. Characterization of the phosphoserine specific antisera is described in the following examples. The specificity of the

polyclonal antibodies for phosphorylated pathway-restricted Smad proteins and not nonphosphorylated pathway-restricted Smad proteins can be tested by methods well known to one of ordinary skill in the art, including immunoprecipitation and Western blotting. Antisera to phosphorylated pathway-restricted Smad proteins which crossreact with nonphosphorylated pathway-restricted Smad proteins can be affinity purified, if desired, to obtain polyclonal antibodies which recognize only phosphorylated pathway-restricted Smad proteins. Alternatively, monoclonal antibodies to phosphorylated pathway-restricted Smad proteins can be prepared as described below in Example 12. Each hybridoma clone producing monoclonal antibodies can be tested for expression of antibodies which recognize selectively the phosphorylated form of pathway-restricted Smad proteins according to standard methods.

Example 10: Pathway-restricted Smad phospho tail antibodies recognize TGF- β superfamily type I receptor-mediated phosphorylated pathway-restricted Smads by Western blot analysis.

COS cells are transfected as described in Example 7 with TGF- β superfamily type I and type II receptors and a pathway-restricted Smad expression plasmid (Smad1, Smad3, Smad5, or Smad9), in the absence or the presence of different amounts of Smad7 expression plasmid. For testing antibodies which recognize phosphorylated Smad1, Smad5 or Smad9, cells are transfected with BMP type I and type II receptors and a Smad1, Smad5 or Smad9 expression plasmid. For testing antibodies which recognize phosphorylated Smad3, cells are transfected with TGF- β or activin type I and type II receptors and a Smad3 expression plasmid. Cells then are treated with or without TGF- β , activin or BMP molecules according to the receptors transfected, and cell lysates are prepared and are used for Western blotting using pathway specific Smad phospho tail antisera according to standard procedures. In the presence of TGF- β , activin or BMP molecules, the pathway specific Smads are phosphorylated and recognized by the phospho tail antibodies. Antibodies which are selective for individual phosphorylated Smad proteins can be identified by blocking experiments using the various peptides against which the antibodies are raised (and non-phosphorylated peptides). For example, an antibody raised against a Smad1 phosphopeptide which does not bind the cognate antigen in the presence of excess Smad1 phosphopeptide, but does bind the cognate antigen in the presence of nonphosphorylated Smad1 peptide or Smad3, Smad5 and/or Smad9 phosphopeptides is an antibody which selectively binds Smad1 phosphorylated at the C-terminus. Therefore the pathway restricted Smad phospho tail antibodies recognize TGF- β type I receptor-mediated

phosphorylated pathway specific Smads.

Example 11: *Pathway specific Smad phospho tail antibody recognizes phosphorylated pathway specific Smads in extracts from metabolically labeled and TGF- β stimulated cells.*

5 Cells lines which overexpress pathway restricted Smads (e.g., naturally overexpress, or which are transfected with expression plasmids) are metabolically labeled with ^{35}S -methionine and ^{35}S -cysteine for 4 hours. During the last hour of labeling, cells are incubated with or without 10 ng/ml TGF- β 1. Cells are lysed according to standard procedures. Cell lysates are precleaned by incubation with protein A-Sepharose beads. After centrifugation to remove the beads, the cell
10 extracts are subjected to immunoprecipitation using the pathway specific Smad phospho tail antisera. To show specificity of the antisera, controls are included containing the respective pathway specific Smad peptides to which the antisera have been raised. Incubations are done for 2 hours, after which the immunocomplexes are collected on protein-A Sepharose beads. The beads are washed three times with lysis buffer (125 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM
15 EDTA, 1 mM PMSF, 1.5% trasytol, and 1% Triton X-100), three times with RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 0.5% Triton X-100, 50 mM Tris-HCl pH 8.0), and three times with high salt buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1% Triton X-100). Immunocomplexes are separated on a 8.5% SDS-PAGE gel and visualized using a Fuji-X
BioImager. Upon treatment of the cells for one hour with 10 ng/ml of TGF- β 1, pathway specific
20 Smad phospho tail antibodies clearly and specifically recognize phosphorylated pathway specific Smad proteins. Therefore the antibodies effectively immunoprecipitate the respective pathway specific Smad proteins present in extracts from metabolically labeled cells.

Example 12: *Preparation of monoclonal antibodies*

25 **1. Immunization of mice**

 Mice (e.g., Balb/c female; Jackson Laboratories, Bar Harbor, ME) are immunized by subcutaneous and/or intraperitoneal injection with a pathway-restricted Smad peptide having one or more C-terminal phosphorylated serine residues suspended in Dulbecco's phosphate buffered saline, then emulsified with an equal volume of complete Freund's adjuvant (Sigma Chemical
30 Co., St. Louis, MO). The mice are given two intraperitoneal booster immunizations of phosphorylated Smad peptide suspended in Dulbecco's phosphate buffered saline (GIBCO, Grand Island, NY), then emulsified with an equal volume of incomplete Freund's adjuvant

(Sigma Chemical Co., St. Louis, MO) at 14 day intervals following the initial immunization.

2. Screening of mice for antibody production

The presence of antibodies to phosphorylated Smad peptide is tested by immunoprecipitation or Western blotting according to standard procedures. For example, ten days following the third and final immunization, a small amount of blood is collected by retro-orbital bleed from each mouse and is clotted. A 1:1000 dilution of each of the serum samples collected from the immunized mice (50µl) is added to a sample of labeled phosphorylated Smad peptide (labeling can be, e.g., ³²P phosphorylation, biotin conjugation, inclusion of a Flag antibody tag, etc.), mixed well and incubated for 60 minutes at 4°C. Protein A-Sepharose is added to the reaction and incubated for 30 min at 4°C. The bound antibody is recovered by centrifugation. Pellets are washed, and SDS-PAGE sample buffer added followed by boiling to disrupt immune complexes. The samples are then analyzed by SDS-PAGE and autoradiography (for radioactive labeling) or other detection method such as chemiluminescence. Alternatively, radioactivity in the washed pellets is measured by scintillation counting. The mouse sera exhibiting the highest degree of binding to phosphorylated Smad peptide is selected for cell fusion to create the monoclonal antibodies.

3. Preparation of hybridomas

Hybridoma cell lines are prepared according to standard protocols; one example is provided below. Briefly, after the mouse with the best antibody titre to the phosphorylated Smad peptide is selected, it is rested for a total of 4 weeks after its last immunization. The mouse is then boosted with phosphorylated Smad peptide by intraperitoneal injection in Dulbecco's phosphate buffered saline. Four days later, the mouse is euthanized by cervical dislocation and the spleen is removed and teased apart into a cell suspension and washed in Dulbecco's phosphate buffered saline. The spleen cells are counted and mixed with SP 2/0 myeloma cells (ATCC Accession No. CRL8006, Rockville, MD) that are incapable of secreting either heavy or light immunoglobulin chains (Kearney et al., *J. Immunology*, 123:1548, 1979) at a ratio of 2:1 (spleen cell:myeloma cells) and then fused using polyethylene glycol 1450 (ATCC, Rockville, MD) according to the standard procedure developed by Kohler and Milstein (*Nature*, 256:495, 1975) in eight 96-well tissue culture plates in selective HAT medium.

Between 10 and 21 days after fusion, hybridoma colonies become visible and are screened by immunoprecipitation, as described above. Alternatively, ELISA assays or other immunobinding assay can be used to determine which hybridomas produce antibodies that bind

phosphorylated Smad peptide. All hybridoma colonies that give a positive response are expanded to 24-well cultures and subcloned by limiting dilution to produce monoclonal cell lines. At this point, additional screening is done with the hybridomas to identify which hybridoma produces an anti-Smad protein antibody. Culture media harvested from hybridoma
5 cultures (supernatants) are screened as described above, e.g. by ELISA (enzyme-linked immunoadsorbent assay) to identify positive clones.

The monoclonal antibodies optionally are examined to determine the subclass of the antibody using an typing kit such as an ISOSTrip Kit (Boehringer Mannheim, Indianapolis, IN). A small aliquot (e.g., 5 µl) of hybridoma supernatant is diluted in PBS and added to a test tube
10 containing blue latex beads bound to anti-mouse Ig antibodies. An isotyping strip is then placed in each tube and the bead/antibody solution moves up the strip by capillary action until the solution passes an antibody bound line containing antibodies specific for the different isotopes. A blue line appears in the area of the strip for each isotype detected in the hybridoma supernatant.

15

Example 13: Separation and sequencing of the heavy and light chains of anti-Smad monoclonal antibody

The antibody may be isolated from the hybridomas and purified by any method known in the art. At least two methods may be used to separate the heavy and light chains of the purified
20 antibody for sequence determination. The first method employs a semi-preparative SDS-PAGE followed by electroblotting onto a polyvinylidene difluoride (PVDF) membrane. Briefly, the purified antibody is subjected to slab gel electrophoresis in SDS after reduction with 2-mercaptoethanol. The resolved heavy and light chains are then transferred onto a membrane such as an IMMOBILON® membrane (a PVDF membrane from Millipore, Bedford, MA) using the
25 electroblotting method of Matsudaira (*J. Biol. Chem.* 261:10035, 1987). Bands corresponding to the heavy and light chains which are identified with Coomassie Brilliant Blue staining may then be excised from the membrane and processed for N-terminal sequencing.

A second more complicated method permits larger amounts of the heavy and light chains to be isolated in solution. This method involves a dialysis step in which the purified antibody
30 sample is dialyzed against 0.1M Tris-HCl, 1 mM EDTA, pH 8.0, at 4°C and then subjected to oxidative sulfitolysis in NaSO₃Na₂S₂O₆, essentially as described by Morehead et al. (*Biochemistry* 23:2500, 1984). Following sulfitolysis, the antibody preparation is dialyzed

against 1M acetic acid, lyophilized to dryness, reconstituted in 1M acetic acid, and subjected to gel filtration in a SEPHADEX G-75 column in 1M acetic acid. The purity of the heavy and light chains following this step can then be assessed by analytical SDS-PAGE and then concentrated for sequencing.

- 5 N-terminal amino acid sequencing may be performed using any commercial amino acid sequencer such as an Applied Biosystems Model 477A protein-peptide sequencer. Analysis of the isolated chains is performed following the instructions of the manufacturer of the sequencer.

10 **Example 14: Oligonucleotide primer design and cloning of Smad phospho tail monoclonal antibody nucleic acids**

1. Preparation of Oligonucleotides

- Based upon the information which is obtained from the foregoing amino acid sequence analyses, degenerate oligonucleotide primers can be designed for use in PCR. Other non-degenerate primers may be designed based upon nucleotide sequence information obtained
15 following PCR amplification of cDNA encoding the complete heavy and light chains.

Oligonucleotide primers are synthesized by standard methods using a commercially available sequencer such as an Applied Biosystems Model 380B Synthesizer.

- Alternatively, PCR amplification of the IgG₁ Fd heavy chain fragments and light chains may be performed using the individual heavy and light chain variable region gene families, and
20 3' constant region primers for IgG₁, k or l as previously described (Kang et al., in *Methods, A Companion to Methods in Enzymology: Vol. 2*, R.A. Lerner and D.R. Burton, ed. Academic Press, NY, pp 111-118, 1991). Primers may contain restriction enzyme sites to allow the sequential ligation of Fd and light chain libraries for various other recombinant uses into a phage display vector.

25 *2. PCR Amplification and DNA Sequencing of Heavy and Light Chains*

- Total cytoplasmic RNA is isolated from the hybridoma cell lines by any method known in the art. First strand cDNA is synthesized directly from total cytoplasmic RNA using reverse transcriptase. Polymerase chain reaction (PCR) amplifications then are carried out according to standard protocols using a thermal cycler or similar equipment. After amplification aliquots of
30 the PCR mixtures are subjected to electrophoresis in agarose gels containing ethidium bromide. The PCR fragments of interest are excised from the gels and purified by e.g. electroelution. The gel-purified PCR fragments are digested with appropriate restriction enzymes and ligated to a

cloning vector such as pBluescript. Competent bacterial cells are transformed with the ligation mixture, grown, and lysed for preparation of DNA. Plasmid DNA is purified by any technique known in the art for purifying DNA, such as the Qiagen plasmid maxiprep kit (Qiagen, Chatsworth, CA). Sequencing is then performed on an automated DNA sequencer. Derived
5 sequences for heavy chain Fd fragments and light chains can then be aligned using various commercially available software packages and the Genbank database.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine
10 experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

A Sequence Listing is presented followed by what is claimed:

SEQUENCE LISTING

(1) GENERAL INFORMATION

- 5 (i) APPLICANT:
(A) NAME: LUDWIG INSTITUTE FOR CANCER RESEARCH
(B) STREET: 1345 AVENUE OF THE AMERICAS
(C) CITY: NEW YORK
(D) STATE: NEW YORK
10 (E) COUNTRY: UNITED STATES OF AMERICA
(F) POSTAL CODE: 10105
- (ii) TITLE OF THE INVENTION: SMAD2 PHOSPHORYLATION AND INTERACTION
WITH SMAD4
15
- (iii) NUMBER OF SEQUENCES: 15
- (iv) CORRESPONDENCE ADDRESS:
20 (A) ADDRESSEE: WOLF, GREENFIELD & SACKS, P.C.
(B) STREET: 600 ATLANTIC AVENUE
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: UNITED STATES OF AMERICA
25 (F) POSTAL CODE: 02210
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
30 (C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
35 (B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/047,807
40 (B) FILING DATE: 20-MAY-1997
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/081,313
(B) FILING DATE: 10-APR-1998
45
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: John R. Van Amsterdam
(B) REGISTRATION NUMBER: 40,212

(C) REFERENCE/DOCKET NUMBER: L0461/7036

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 617-720-3500

(B) TELEFAX: 617-720-2441

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1640 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTISENSE: NO

(ix) FEATURE:

(A) NAME/KEY: Coding Sequence

(B) LOCATION: 60...1460

(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCCGAGGGT AGATTTACCG GGCTTTTCT GAGTGTGGAT TGTTCCTTTT GGTAAGAAA	59
ATG TCG TCC ATC TTG CCA TTC ACT CCG CCA GTG GTG AAG AGA CTT CTG	107
Met Ser Ser Ile Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu	
1 5 10 15	
GGA TGG AAA AAA TCA GCC GGT GGG TCT GGA GGA GCA GGT GGT GGA GAG	155
Gly Trp Lys Lys Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Gly Glu	
20 25 30	
CAG AAT GGA CAG GAA GAA AAG TGG TGT CAA AAA GCA GTG AAA AGT CTG	203
Gln Asn Gly Gln Glu Glu Lys Trp Cys Gln Lys Ala Val Lys Ser Leu	
35 40 45	
GTG AAA AAG CTA AAG AAA ACA GGA CGG TTA GAT GAG CTT GAG AAA GCC	251
Val Lys Lys Leu Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala	
50 55 60	
ATC ACC ACT CAG AAT TGC AAT ACT AAA TGT GTC ACC ATA CCA AGC ACT	299
Ile Thr Thr Gln Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr	
65 70 75 80	
TGC TCT GAA ATT TGG GGA CTG AGT ACA GCA AAT ACG GTA GAT CAG TGG	347

	Cys	Ser	Glu	Ile	Trp	Gly	Leu	Ser	Thr	Ala	Asn	Thr	Val	Asp	Gln	Trp	
					85					90					95		
	GAC	ACA	ACA	GGC	CTT	TAC	AGC	TTC	TCT	GAA	CAA	ACC	AGG	TCT	CTT	GAT	395
5	Asp	Thr	Thr	Gly	Leu	Tyr	Ser	Phe	Ser	Glu	Gln	Thr	Arg	Ser	Leu	Asp	
				100					105					110			
	GGC	CGT	CTT	CAG	GTT	TCA	CAC	CGG	AAA	GGG	TTG	CCA	CAT	GTT	ATA	TAT	443
10	Gly	Arg	Leu	Gln	Val	Ser	His	Arg	Lys	Gly	Leu	Pro	His	Val	Ile	Tyr	
			115					120					125				
	TGC	CGG	CTC	TGG	CGC	TGG	CCG	GAC	CTT	CAC	AGT	CAT	CAT	GAG	CTC	AAG	491
	Cys	Arg	Leu	Trp	Arg	Trp	Pro	Asp	Leu	His	Ser	His	His	Glu	Leu	Lys	
15			130				135					140					
	GCA	ATC	GAA	AAC	TGC	GAA	TAT	GCT	TTT	AAT	CTG	AAA	AAA	GAT	GAA	GTG	539
	Ala	Ile	Glu	Asn	Cys	Glu	Tyr	Ala	Phe	Asn	Leu	Lys	Lys	Asp	Glu	Val	
	145				150					155					160		
20	TGT	GTA	AAT	CCG	TAC	CAC	TAC	CAG	AGA	GTT	GAG	ACC	CCA	GTC	TTG	CCT	587
	Cys	Val	Asn	Pro	Tyr	His	Tyr	Gln	Arg	Val	Glu	Thr	Pro	Val	Leu	Pro	
				165					170					175			
	CCA	GTC	TTA	GTG	CCT	CGG	CAC	ACG	GAG	ATT	CTA	ACA	GAA	CTG	CCG	CCC	635
25	Pro	Val	Leu	Val	Pro	Arg	His	Thr	Glu	Ile	Leu	Thr	Glu	Leu	Pro	Pro	
			180					185					190				
	CTG	GAT	GAC	TAC	ACC	CAC	TCC	ATT	CCA	GAA	AAC	ACA	AAT	TTC	CCA	GCA	683
30	Leu	Asp	Asp	Tyr	Thr	His	Ser	Ile	Pro	Glu	Asn	Thr	Asn	Phe	Pro	Ala	
		195					200					205					
	GGA	ATT	GAG	CCA	CAG	AGT	AAT	TAC	ATC	CCA	GAA	ACA	CCA	CCA	CCT	GGA	731
	Gly	Ile	Glu	Pro	Gln	Ser	Asn	Tyr	Ile	Pro	Glu	Thr	Pro	Pro	Pro	Gly	
35		210				215				220							
	TAT	ATC	AGT	GAA	GAT	GGA	GAA	ACA	AGT	GAC	CAA	CAG	TTG	AAC	CAA	AGT	779
	Tyr	Ile	Ser	Glu	Asp	Gly	Glu	Thr	Ser	Asp	Gln	Gln	Leu	Asn	Gln	Ser	
	225				230				235				240				
40	ATG	GAC	ACA	GGC	TCT	CCG	GCT	GAA	CTG	TCT	CCT	ACT	ACT	CTC	TCT	CCT	827
	Met	Asp	Thr	Gly	Ser	Pro	Ala	Glu	Leu	Ser	Pro	Thr	Thr	Leu	Ser	Pro	
				245				250					255				
	GTT	AAT	CAC	AGC	TTG	GAT	TTG	CAG	CCA	GTT	ACT	TAC	TCG	GAA	CCT	GCA	875
45	Val	Asn	His	Ser	Leu	Asp	Leu	Gln	Pro	Val	Thr	Tyr	Ser	Glu	Pro	Ala	
			260				265					270					
	TTC	TGG	TGT	TCA	ATC	GCA	TAC	TAT	GAA	CTA	AAC	CAG	AGG	GTT	GGA	GAG	923

	Phe Trp Cys Ser Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu	
	275 280 285	
5	ACC TTC CAT GCG TCA CAG CCC TCG CTC ACT GTA GAC GGC TTC ACA GAC Thr Phe His Ala Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp	971
	290 295 300	
10	CCA TCA AAC TCG GAG AGG TTC TGC TTA GGC TTG CTC TCC AAC GTT AAC Pro Ser Asn Ser Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn	1019
	305 310 315 320	
15	CGA AAT GCC ACT GTA GAA ATG ACA AGA AGA CAT ATA GGA AGG GGA GTG Arg Asn Ala Thr Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val	1067
	325 330 335	
20	CGC TTG TAT TAC ATA GGT GGG GAA GTG TTT GCT GAG TGC CTA AGT GAT Arg Leu Tyr Tyr Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp	1115
	340 345 350	
25	AGT GCA ATC TTT GTG CAG AGC CCC AAC TGT AAC CAG AGA TAC GGC TGG Ser Ala Ile Phe Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp	1163
	355 360 365	
30	CAC CCT GCA ACA GTG TGT AAG ATC CCA CCA GGC TGT AAC CTG AAG ATC His Pro Ala Thr Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile	1211
	370 375 380	
35	TTC AAC AAC CAA GAA TTT GCT GCT CTT CTG GCT CAG TCT GTC AAC CAG Phe Asn Asn Gln Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln	1259
	385 390 395 400	
40	GGT TTT GAA GCC GTT TAT CAG CTA ACC CGA ATG TGC ACC ATA AGA ATG Gly Phe Glu Ala Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met	1307
	405 410 415	
45	AGT TTT GTG AAG GGC TGG GGA GCA GAA TAT CGG AGG CAG ACA GTA ACA Ser Phe Val Lys Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr	1355
	420 425 430	
50	AGT ACT CCT TGC TGG ATT GAA CTT CAT CTG AAT GGC CCT CTG CAG TGG Ser Thr Pro Cys Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp	1403
	435 440 445	
55	CTG GAC AAA GTA TTA ACT CAG ATG GGA TCC CCT TCA GTG CGA TGC TCA Leu Asp Lys Val Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser	1451
	450 455 460	
60	AGC ATG TCG TAAACCCATC AAAGACTCGC TGTAACAGCT CCTCCGTCGT AGTATTCAT	1509

	GTATGATCCC GTGGACTGTT TGCTATCCAA AAATTCCAGA GCAAAAACAG CACTTGAGGT	1569
5	CTCATCAGTT AAAGCACCTT GTGGAATCTG TTTCTATAT TTGAATATTA GATGGGAAAA	1629
	TTAGTGTCTA	1639

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 467 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

20	Met	Ser	Ser	Ile	Leu	Pro	Phe	Thr	Pro	Pro	Val	Val	Lys	Arg	Leu	Leu
	1				5					10					15	
	Gly	Trp	Lys	Lys	Ser	Ala	Gly	Gly	Ser	Gly	Gly	Ala	Gly	Gly	Gly	Glu
				20					25					30		
25	Gln	Asn	Gly	Gln	Glu	Glu	Lys	Trp	Cys	Gln	Lys	Ala	Val	Lys	Ser	Leu
			35					40					45			
	Val	Lys	Lys	Leu	Lys	Lys	Thr	Gly	Arg	Leu	Asp	Glu	Leu	Glu	Lys	Ala
		50					55					60				
	Ile	Thr	Thr	Gln	Asn	Cys	Asn	Thr	Lys	Cys	Val	Thr	Ile	Pro	Ser	Thr
30	65					70					75					80
	Cys	Ser	Glu	Ile	Trp	Gly	Leu	Ser	Thr	Ala	Asn	Thr	Val	Asp	Gln	Trp
					85					90					95	
	Asp	Thr	Thr	Gly	Leu	Tyr	Ser	Phe	Ser	Glu	Gln	Thr	Arg	Ser	Leu	Asp
				100					105					110		
35	Gly	Arg	Leu	Gln	Val	Ser	His	Arg	Lys	Gly	Leu	Pro	His	Val	Ile	Tyr
			115						120					125		
	Cys	Arg	Leu	Trp	Arg	Trp	Pro	Asp	Leu	His	Ser	His	His	Glu	Leu	Lys
			130					135				140				
	Ala	Ile	Glu	Asn	Cys	Glu	Tyr	Ala	Phe	Asn	Leu	Lys	Lys	Asp	Glu	Val
40	145					150					155					160
	Cys	Val	Asn	Pro	Tyr	His	Tyr	Gln	Arg	Val	Glu	Thr	Pro	Val	Leu	Pro
					165					170					175	
	Pro	Val	Leu	Val	Pro	Arg	His	Thr	Glu	Ile	Leu	Thr	Glu	Leu	Pro	Pro
			180						185					190		
45	Leu	Asp	Asp	Tyr	Thr	His	Ser	Ile	Pro	Glu	Asn	Thr	Asn	Phe	Pro	Ala
			195					200					205			
	Gly	Ile	Glu	Pro	Gln	Ser	Asn	Tyr	Ile	Pro	Glu	Thr	Pro	Pro	Pro	Gly
			210				215					220				

Tyr Ile Ser Glu Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser
 225 230 235 240
 Met Asp Thr Gly Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro
 245 250 255
 5 Val Asn His Ser Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala
 260 265 270
 Phe Trp Cys Ser Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu
 275 280 285
 Thr Phe His Ala Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp
 10 290 295 300
 Pro Ser Asn Ser Glu Arg Phe Cys Leu Gly Leu Ser Asn Val Asn
 305 310 315 320
 Arg Asn Ala Thr Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val
 325 330 335
 15 Arg Leu Tyr Tyr Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp
 340 345 350
 Ser Ala Ile Phe Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp
 355 360 365
 His Pro Ala Thr Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile
 20 370 375 380
 Phe Asn Asn Gln Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln
 385 390 395 400
 Gly Phe Glu Ala Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met
 405 410 415
 25 Ser Phe Val Lys Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr
 420 425 430
 Ser Thr Pro Cys Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp
 435 440 445
 Leu Asp Lys Val Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser
 30 450 455 460
 Ser Met Ser
 465

(2) INFORMATION FOR SEQ ID NO:3:

35

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

45

Ser Ser Met Ser

1

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser Ser Met Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:PEPTIDE

(ix) FEATURE:

- (A) NAME/KEY: Other
(B) LOCATION: 3
(D) OTHER INFORMATION: Xaa = Met or Val

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Ser Xaa Ser
1

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE:PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Lys Lys Ser Ser Met Ser
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Lys Lys Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser Ser Met
1 5 10 15
Ser

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Ser Val Ser
1

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Lys Lys Ser Ser Val Ser

1 5

(2) INFORMATION FOR SEQ ID NO:10:

5 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

15 Thr Gln Met Gly Ser Pro His Asn Pro Ile Ser Ser Val Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

20 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

30 Thr Gln Met Gly Ser Pro Ser Ile Arg Cys Ser Ser Val Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

40

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

45 Thr Gln Met Gly Ser Pro Leu Asn Pro Ile Ser Ser Val Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: PEPTIDE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Thr Gln Met Gly Ser Pro His Asn Pro Ile Ser Ser Val Ser
 1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1710 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
 (B) LOCATION: 107...1507
 (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCGCGGCGGC GGAGAAGCAG CTCGCCAGCC AGCAGCCCGC CAGCCGCGG GAGGTTTCGAT 60
 ACAAGAGGCT GTTTTCCTAG CGTGGCTTGC TGCCTTTGGT AAGAAC ATG TCG TCC 115
 Met Ser Ser
 1
 ATC TTG CCA TTC ACG CCG CCA GTT GTG AAG AGA CTG CTG GGA TGG AAG 163
 Ile Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu Gly Trp Lys
 5 10 15
 AAG TCA GCT GGT GGG TCT GGA GGA GCA GGC GGA GGA GAG CAG AAT GGG 211
 Lys Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Gly Glu Gln Asn Gly
 20 25 30 35
 CAG GAA GAA AAG TGG TGT GAG AAA GCA GTG AAA AGT CTG GTG AAG AAG 259
 Gln Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu Val Lys Lys
 40 45 50

	CTA AAG AAA ACA CGA CGA TTA GAT GAG CTT GAG AAA GCC ATC ACC ACT	307
	Leu Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala Ile Thr Thr	
	55 60 65	
5	CAA AAC TGT AAT ACT AAA TGT GTT ACC ATA CCA AGC ACT TGC TCT GAA	355
	Gln Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr Cys Ser Glu	
	70 75 80	
	ATT TGG GGA CTG AGT ACA CCA AAT ACG ATA GAT CAG TGG GAT ACA ACA	403
10	Ile Trp Gly Leu Ser Thr Pro Asn Thr Ile Asp Gln Trp Asp Thr Thr	
	85 90 95	
	GGC CTT TAC AGC TTC TCT GAA CAA ACC AGG TCT CTT GAT GGT CGT CTC	451
	Gly Leu Tyr Ser Phe Ser Glu Gln Thr Arg Ser Leu Asp Gly Arg Leu	
15	100 105 110 115	
	CAG GTA TCC CAT CGA AAA GGA TTG CCA CAT GTT ATA TAT TGC CGA TTA	499
	Gln Val Ser His Arg Lys Gly Leu Pro His Val Ile Tyr Cys Arg Leu	
	120 125 130	
20	TGG CGC TGG CCT GAT CTT CAC AGT CAT CAT GAA CTC AAG GCA ATT GAA	547
	Trp Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys Ala Ile Glu	
	135 140 145	
25	AAC TGC GAA TAT GCT TTT AAT CTT AAA AAG GAT GAA GTA TGT GTA AAC	595
	Asn Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val Cys Val Asn	
	150 155 160	
	CCT TAC CAC TAT CAG AGA GTT GAG ACA CCA GTT TTG CCT CCA GTA TTA	643
30	Pro Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro Pro Val Leu	
	165 170 175	
	GTG CCC CGA CAC ACC GAG ATC CTA ACA GAA CTT CCG CCT CTG GAT GAC	691
	Val Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro Leu Asp Asp	
35	180 185 190 195	
	TAT ACT CAC TCC ATT CCA GAA AAC ACT AAC TTC CCA GCA GGA ATT GAG	739
	Tyr Thr His Ser Ile Pro Glu Asn Thr Asn Phe Pro Ala Gly Ile Glu	
	200 205 210	
40	CCA CAG AGT AAT TAT ATT CCA GAA ACG CCA CCT CCT GGA TAT ATC AGT	787
	Pro Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Pro Gly Tyr Ile Ser	
	215 220 225	
45	GAA GAT GGA GAA ACA AGT GAC CAA CAG TTG AAT CAA AGT ATG GAC ACA	835
	Glu Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser Met Asp Thr	
	230 235 240	

	GGC TCT CCA GCA GAA CTA TCT CCT ACT ACT CTT TCC CCT GTT AAT CAT	883
	Gly Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro Val Asn His	
	245 250 255	
5	AGC TTG GAT TTA CAG CCA GTT ACT TAC TCA GAA CCT GCA TTT TGG TGT	931
	Ser Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala Phe Trp Cys	
	260 265 270 275	
10	TCA ATA GCA TAT TAT GAA TTA AAT CAG AGG GTT GGA GAA ACC TTC CAT	979
	Ser Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu Thr Phe His	
	280 285 290	
15	GCA TCA CAG CCC TCA CTC ACT GTA GAT GGC TTT ACA GAC CCA TCA AAT	1027
	Ala Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp Pro Ser Asn	
	295 300 305	
20	TCA GAG AGG TTC TGC TTA GGT TTA CTC TCC AAT GTT AAC CGA AAT GCC	1075
	Ser Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn Arg Asn Ala	
	310 315 320	
25	ACG GTA GAA ATG ACA AGA AGG CAT ATA GGA AGA GGA GTG CGC TTA TAC	1123
	Thr Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val Arg Leu Tyr	
	325 330 335	
30	TAC ATA GGT GGG GAA GTT TTT GCT GAG TGC CTA AGT GAT AGT GCA ATC	1171
	Tyr Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp Ser Ala Ile	
	340 345 350 355	
35	TTT GTG CAG AGC CCC AAT TGT AAT CAG AGA TAT GGC TGG CAC CCT GCA	1219
	Phe Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp His Pro Ala	
	360 365 370	
40	ACA GTG TGT AAA ATT CCA CCA GGC TGT AAT CTG AAG ATC TTC AAC AAC	1267
	Thr Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile Phe Asn Asn	
	375 380 385	
45	CAG GAA TTT GCT GCT CTT CTG GCT CAG TCT GTT AAT CAG GGT TTT GAA	1315
	Gln Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln Gly Phe Glu	
	390 395 400	
50	GCC GTC TAT CAG CTA ACT AGA ATG TGC ACC ATA AGA ATG AGT TTT GTG	1363
	Ala Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met Ser Phe Val	
	405 410 415	
55	AAA GGG TGG GGA GCA GAA TAC CGA AGG CAG ACG GTA ACA AGT ACT CCT	1411
	Lys Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr Ser Thr Pro	
	420 425 430 435	

TGC TGG ATT GAA CTT CAT CTG AAT GGA CCT CTA CAG TGG TTG GAC AAA 1459
 Cys Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp Leu Asp Lys
 440 445 450

5 GTA TTA ACT CAG ATG GGA TCC CCT TCA GTG CGT TGC TCA AGC ATG TCA T 1508
 Val Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser Ser Met Ser
 455 460 465

AAAGCTTCAC CAATCAAGTC CCATGAAAAG ACTTAATGTA ACAACTCTTC TGTCATAGCA 1568
 10 TTGTGTGTGG TCCCTATGGA CTGTTTACTA TCCAAAAGTT CAAGAGAGAA AACAGCACTT 1628
 GAGGTCTCAT CAATTAAAGC ACCTGTGGA ATCTGTTTCC TATATTTGAA TATTAGATGG 1688
 GAAAATTAGT GTCATAAAGA TC 1710

(2) INFORMATION FOR SEQ ID NO:15:

15

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 467 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

20

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Ser Ser Ile Leu Pro Phe Thr Pro Pro Val Val Lys Arg Leu Leu
 1 5 10 15

30 Gly Trp Lys Lys Ser Ala Gly Gly Ser Gly Gly Ala Gly Gly Gly Glu
 20 25 30

Gln Asn Gly Gln Glu Glu Lys Trp Cys Glu Lys Ala Val Lys Ser Leu
 35 40 45

Val Lys Lys Leu Lys Lys Thr Gly Arg Leu Asp Glu Leu Glu Lys Ala
 35 50 55 60

Ile Thr Thr Gln Asn Cys Asn Thr Lys Cys Val Thr Ile Pro Ser Thr
 65 70 75 80

Cys Ser Glu Ile Trp Gly Leu Ser Thr Pro Asn Thr Ile Asp Gln Trp
 85 90 95

40 Asp Thr Thr Gly Leu Tyr Ser Phe Ser Glu Gln Thr Arg Ser Leu Asp
 100 105 110

Gly Arg Leu Gln Val Ser His Arg Lys Gly Leu Pro His Val Ile Tyr
 115 120 125

Cys Arg Leu Trp Arg Trp Pro Asp Leu His Ser His His Glu Leu Lys
 45 130 135 140

Ala Ile Glu Asn Cys Glu Tyr Ala Phe Asn Leu Lys Lys Asp Glu Val
 145 150 155 160

Cys Val Asn Pro Tyr His Tyr Gln Arg Val Glu Thr Pro Val Leu Pro

165 170 175
 Pro Val Leu Val Pro Arg His Thr Glu Ile Leu Thr Glu Leu Pro Pro
 180 185 190
 Leu Asp Asp Tyr Thr His Ser Ile Pro Glu Asn Thr Asn Phe Pro Ala
 195 200 205
 5 Gly Ile Glu Pro Gln Ser Asn Tyr Ile Pro Glu Thr Pro Pro Gly
 210 215 220
 Tyr Ile Ser Glu Asp Gly Glu Thr Ser Asp Gln Gln Leu Asn Gln Ser
 225 230 235 240
 10 Met Asp Thr Gly Ser Pro Ala Glu Leu Ser Pro Thr Thr Leu Ser Pro
 245 250 255
 Val Asn His Ser Leu Asp Leu Gln Pro Val Thr Tyr Ser Glu Pro Ala
 260 265 270
 Phe Trp Cys Ser Ile Ala Tyr Tyr Glu Leu Asn Gln Arg Val Gly Glu
 275 280 285
 15 Thr Phe His Ala Ser Gln Pro Ser Leu Thr Val Asp Gly Phe Thr Asp
 290 295 300
 Pro Ser Asn Ser Glu Arg Phe Cys Leu Gly Leu Leu Ser Asn Val Asn
 305 310 315 320
 20 Arg Asn Ala Thr Val Glu Met Thr Arg Arg His Ile Gly Arg Gly Val
 325 330 335
 Arg Leu Tyr Tyr Ile Gly Gly Glu Val Phe Ala Glu Cys Leu Ser Asp
 340 345 350
 Ser Ala Ile Phe Val Gln Ser Pro Asn Cys Asn Gln Arg Tyr Gly Trp
 355 360 365
 25 His Pro Ala Thr Val Cys Lys Ile Pro Pro Gly Cys Asn Leu Lys Ile
 370 375 380
 Phe Asn Asn Gln Glu Phe Ala Ala Leu Leu Ala Gln Ser Val Asn Gln
 385 390 395 400
 30 Gly Phe Glu Ala Val Tyr Gln Leu Thr Arg Met Cys Thr Ile Arg Met
 405 410 415
 Ser Phe Val Lys Gly Trp Gly Ala Glu Tyr Arg Arg Gln Thr Val Thr
 420 425 430
 Ser Thr Pro Cys Trp Ile Glu Leu His Leu Asn Gly Pro Leu Gln Trp
 435 440 445
 35 Leu Asp Lys Val Leu Thr Gln Met Gly Ser Pro Ser Val Arg Cys Ser
 450 455 460
 Ser Met Ser
 465

CLAIMS

1. An isolated Smad2 polypeptide comprising a polypeptide having the amino acid sequence of SEQ ID NO:2 or its human homolog except that the polypeptide includes a mutation comprising a non-serine amino acid located at one or more of amino acids 464, 465 and 467.
5
2. The isolated Smad2 polypeptide of claim 1 wherein the mutation is located at positions selected from the group consisting of 464; 465; 467; 464 and 465; 464 and 467; 465 and 467; and 464, 465 and 467.
- 10 3. The isolated Smad2 polypeptide of claim 1 wherein the mutation is selected from the group consisting of Ser464A; Ser465A; Ser467A; Ser464A and Ser465A; Ser464A and Ser467A; Ser465A and Ser467A; and Ser464A, Ser465A and Ser465A.
4. The isolated Smad2 polypeptide of claim 3, wherein the mutation is Ser464A.
15
5. The isolated Smad2 polypeptide of claim 3, wherein the mutation is Ser465A.
6. The isolated Smad2 polypeptide of claim 3, wherein the mutation is Ser467A.
- 20 7. The isolated Smad2 polypeptide of claim 3, wherein the mutation is Ser464A and Ser465A.
8. The isolated Smad2 polypeptide of claim 3, wherein the mutation is Ser464A and Ser467A.
25
9. The isolated Smad2 polypeptide of claim 3, wherein the mutation is Ser465A and Ser467A.
10. The isolated Smad2 polypeptide of claim 3, wherein the mutation is Ser464A, Ser465A
30 and Ser467A.
11. An isolated nucleic acid molecule which encodes the isolated polypeptide of any of

claims 1-10.

12. An expression vector comprising the isolated nucleic acid molecule of claim 11 operably linked to a promoter.

5

13. A host cell transformed or transfected with the expression vector of claim 12.

14. An isolated Smad4 binding polypeptide comprising the amino acid sequence of SEQ ID NO:3.

10

15. The isolated Smad4 binding polypeptide of claim 14, wherein the isolated polypeptide comprises a polypeptide selected from the group consisting of the 4 most C-terminal amino acids of SEQ ID NO:2, the 5 most C-terminal amino acids of SEQ ID NO:2, the 6 most C-terminal amino acids of SEQ ID NO:2, the 7 most C-terminal amino acids of SEQ ID NO:2, the 8 most C-terminal amino acids of SEQ ID NO:2, the 9 most C-terminal amino acids of SEQ ID NO:2, the 10 most C-terminal amino acids of SEQ ID NO:2, the 11 most C-terminal amino acids of SEQ ID NO:2, the 12 most C-terminal amino acids of SEQ ID NO:2, the 13 most C-terminal amino acids of SEQ ID NO:2, the 14 most C-terminal amino acids of SEQ ID NO:2, the 15 most C-terminal amino acids of SEQ ID NO:2, the 16 most C-terminal amino acids of SEQ ID NO:2, the 17 most C-terminal amino acids of SEQ ID NO:2, the 18 most C-terminal amino acids of SEQ ID NO:2, the 19 most C-terminal amino acids of SEQ ID NO:2, and the 20 most C-terminal amino acids of SEQ ID NO:2.

16. The isolated Smad4 binding polypeptide of claim 15, wherein the isolated polypeptide comprises the amino acid sequence of SEQ ID NO:4.

17. The isolated Smad4 binding polypeptide of claim 14, 15 or 16, wherein the isolated polypeptide is phosphorylated on one or more amino acids selected from the group consisting of Ser464, Ser465, Ser467, Ser464/Ser465, Ser464/Ser467, Ser465/Ser467 and Ser464/Ser465/Ser467.

18. An isolated polypeptide which binds selectively the polypeptide of any of claims 1, 2, 14,

15, 16 or 17 provided that the isolated polypeptide is not T β R-I, T β R-II or Smad4.

19. The isolated polypeptide of claim 18, wherein the isolated polypeptide is an antibody.

5 20. The isolated polypeptide of claim 18, wherein the isolated polypeptide is an antibody fragment selected from the group consisting of a Fab fragment, a F(ab)₂ fragment or a fragment including a CDR3 region selective for a Smad2 polypeptide.

20B. The isolated polypeptide of claim 18, wherein the isolated polypeptide is a monoclonal
10 antibody.

21. A method for inhibiting TGF- β signal transduction in a mammalian cell, comprising contacting the mammalian cell with an amount of an inhibitor of phosphorylation of endogenous Smad2 effective to reduce TGF- β signal transduction in the mammalian cell.

15

22. The method of claim 21, wherein the inhibitor is a dominant negative Smad2 polypeptide.

23. The method of claim 22 wherein the dominant negative Smad2 is the polypeptide of any
20 of claims 1, 2 or 3.

24. A composition comprising:
the polypeptide of claim 1, and
a pharmaceutically acceptable carrier.

25

25. A method for decreasing TGF- β signal transduction activity in a subject comprising administering to a subject in need of such treatment an agent that selectively binds to a TGF- β receptor, in an amount effective to decrease TGF- β signal transduction activity in the subject.

30

26. The method of claim 25, wherein the agent is an isolated polypeptide as claimed in any of claims 1, 2 or 3.

27. A method for identifying lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated with Smad2/TGF- β receptor interaction, comprising forming a mixture comprising a Smad2 polypeptide as claimed in claim 1, a TGF- β receptor, and a candidate pharmacological agent,
- 5 incubating the mixture under conditions which, in the absence of the candidate pharmacological agent, permit a first amount of specific binding of the TGF- β receptor by the Smad2 polypeptide, and
- detecting a test amount of the specific binding of the TGF- β receptor by the Smad2 polypeptide, wherein reduction of the test amount of specific binding of the the Smad2
- 10 polypeptide relative to the first amount of specific binding of the the Smad2 polypeptide indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which disrupts the Smad2/TGF- β receptor interaction, and wherein increase of the test amount of specific binding relative to the first amount of specific binding indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which
- 15 enhances the Smad2/TGF- β receptor interaction.
28. The method of claim 27, wherein the Smad2 polypeptide is the polypeptide of claim 3.
29. A method for identifying lead compounds for a pharmacological agent useful in the
- 20 diagnosis or treatment of disease associated with TGF- β mediated Smad2 signal transduction activity, comprising
- forming a mixture comprising a wild type Smad2 polypeptide, a TGF- β receptor, and a candidate pharmacological agent comprising a mutated Smad2 polypeptide,
- incubating the mixture under conditions which, in the absence of the candidate
- 25 pharmacological agent, permit a first amount of TGF- β mediated phosphorylation of the wild type Smad2 polypeptide, and
- detecting a test amount of TGF- β mediated phosphorylation of the wild type Smad2 polypeptide, wherein reduction of the test amount of the phosphorylation of the wild type Smad2
- polypeptide relative to the first amount of the phosphorylation of the wild type Smad 2
- 30 polypeptide indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which disrupts the TGF- β mediated Smad2 signal transduction activity.

30. The method of claim 29, wherein the mutated Smad2 polypeptide is the polypeptide of claim 3 or TGF- β receptor binding fragments thereof.
31. An isolated polypeptide which binds selectively to at least one pathway-restricted Smad polypeptide or fragment thereof having the C-terminal amino acid sequence of SEQ ID NO:5, wherein the pathway-restricted Smad polypeptide or fragment thereof is phosphorylated on at least one serine residue of SEQ ID NO:5, provided that the isolated polypeptide is not T β R-I, T β R-II or Smad4.
32. The isolated polypeptide of claim 31, wherein the pathway restricted Smad polypeptide is selected from the group consisting of Smad1, Smad3, Smad5 and Smad9.
33. The isolated polypeptide of claim 31, wherein the at least one serine residue is selected from the group consisting of the second serine residue, the third serine residue and the second and third serine residues.
34. The isolated polypeptide of claim 31, wherein the isolated polypeptide is an antibody.
35. The isolated polypeptide of claim 34, wherein the isolated polypeptide is an antibody fragment selected from the group consisting of a Fab fragment, a F(ab)₂ fragment or a fragment including a CDR3 region selective for a pathway-restricted Smad polypeptide.
36. The isolated polypeptide of claim 34, wherein the antibody is a monoclonal antibody.
37. The isolated polypeptide of claim 34, wherein the antibody binds selectively to one of the pathway restricted Smad polypeptides.
38. A method for determining the amount of a pathway-restricted Smad polypeptide having a phosphorylated C-terminal serine residue in a biological sample, comprising
contacting the biological sample with an isolated polypeptide that selectively binds at least one phosphorylated serine of the amino acid sequence as set forth in SEQ ID NO:5,
determining the binding of the isolated polypeptide to the pathway-restricted Smad

polypeptide, and

comparing the binding to a control as a determination of the amount of a pathway-restricted Smad polypeptide having a phosphorylated C-terminal serine residue in the biological sample.

5

39. The method of claim 38, wherein the pathway-restricted Smad polypeptide is selected from the group consisting of Smad1, Smad2, Smad3, Smad5, and Smad9.

40. The method of claim 38, wherein the isolated polypeptide is an antibody.

10

41. The method of claim 40, wherein the antibody selectively binds a pathway-restricted Smad polypeptide having at least one phosphorylated C-terminal serine residue selected from the group consisting of the second serine residue of SEQ ID NO:5, the third serine residue of SEQ ID NO:5 and the second and third serine residues of SEQ ID NO:5.

15

42. A method for identifying lead compounds for a pharmacological agent which modulate phosphorylation of the C-terminal serine residues of a pathway-restricted Smad polypeptide, comprising

forming a mixture comprising a pathway-restricted Smad polypeptide having at least one C-terminal serine residue, a TGF- β superfamily receptor or receptor complex capable of phosphorylating the at least one C-terminal serine residue, and a candidate pharmacological agent,

incubating the mixture under conditions which, in the absence of the candidate pharmacological agent, permit a first amount of phosphorylation of the at least one C-terminal serine residue by the TGF- β superfamily receptor or receptor complex, and

detecting a test amount of the phosphorylation of the at least one C-terminal serine residue by the TGF- β superfamily receptor or receptor complex, wherein reduction of the test amount of phosphorylation relative to the first amount of phosphorylation indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces phosphorylation of the at least one C-terminal serine residue of a pathway-restricted Smad polypeptide, and wherein an increase of the test amount of phosphorylation relative to the first amount of phosphorylation indicates that the candidate pharmacological agent is a lead

30

compound for a pharmacological agent which increases the phosphorylation of the C-terminal serine residues of a pathway-restricted Smad polypeptide.

43. The method of claim 42, wherein the pathway-restricted Smad polypeptide is selected
5 from the group consisting of Smad1, Smad2, Smad3, Smad5, and Smad9.

44. The method of claim 42, wherein the at least one C-terminal serine residue is selected
from the group consisting of the second serine residue of SEQ ID NO:5, the third serine residue
of SEQ ID NO:5 and the second and third serine residues of SEQ ID NO:5.

10

45. The method of claim 42, wherein the step of forming a mixture comprising a pathway-
restricted Smad polypeptide, a TGF- β superfamily receptor or receptor complex, and a candidate
pharmacological agent comprises contacting a cell which includes a pathway-restricted Smad
polypeptide and a TGF- β receptor or receptor complex with a candidate pharmacological agent.

15

46. The method of claim 42 or claim 45, wherein the step of forming a mixture further
comprises adding a ligand which activates the TGF- β superfamily receptor or receptor complex.

47. A method for reducing heteromeric Smad protein complex formation in a cell,
20 comprising

providing an antibody which selectively binds a phosphorylated pathway-restricted Smad
polypeptide or a nucleic acid encoding the antibody, and

contacting the cell with an amount of the antibody or the nucleic acid encoding the
antibody sufficient to reduce formation of the heteromeric Smad protein complex in the cell.

25

48. The method of claim 47, wherein the pathway-restricted Smad polypeptide is selected
from the group consisting of Smad1, Smad2, Smad3, Smad5, and Smad9.

49. The method of claim 47, wherein the antibody selectively binds a pathway-restricted
30 Smad polypeptide having at least one phosphorylated C-terminal serine residue selected from the
group consisting of the second serine residue of SEQ ID NO:5, the third serine residue of SEQ
ID NO:5 and the second and third serine residues of SEQ ID NO:5.

FIG. 1

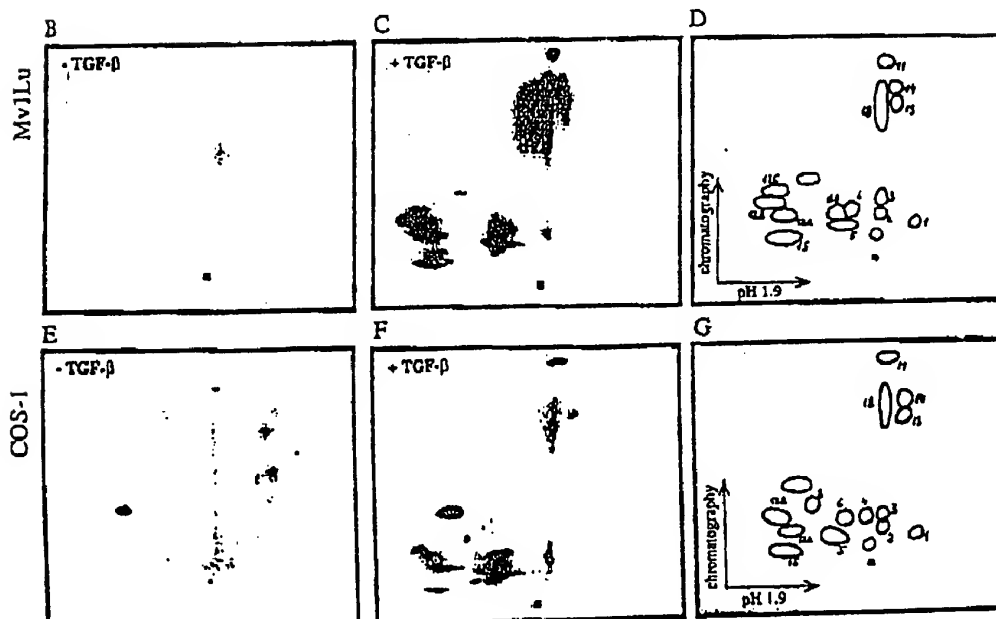
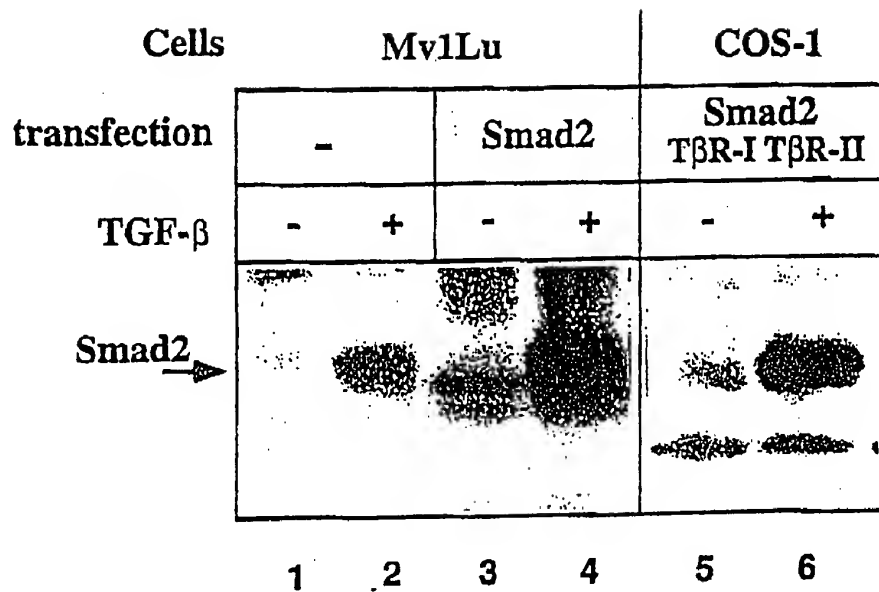


FIG. 2

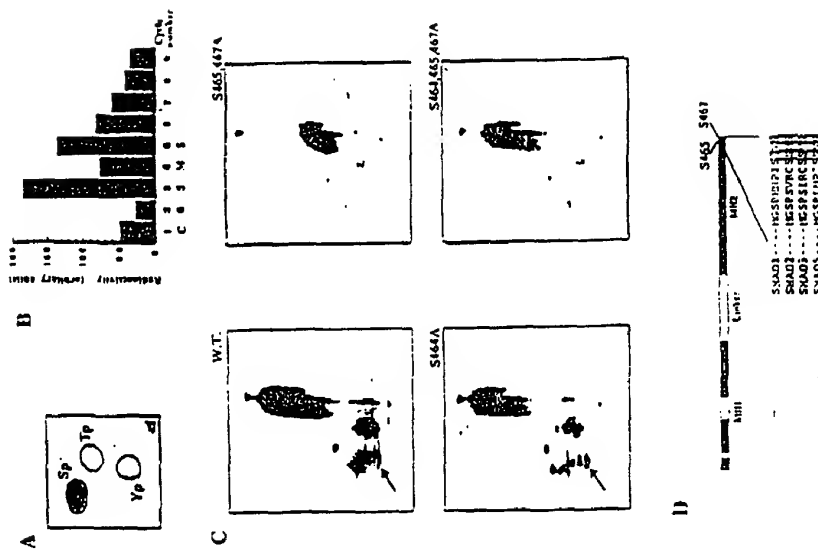


FIG. 3

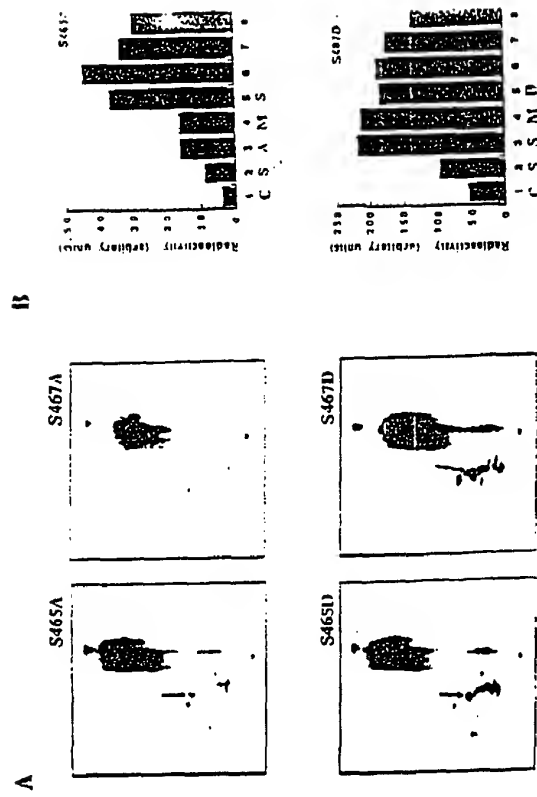


FIG. 4

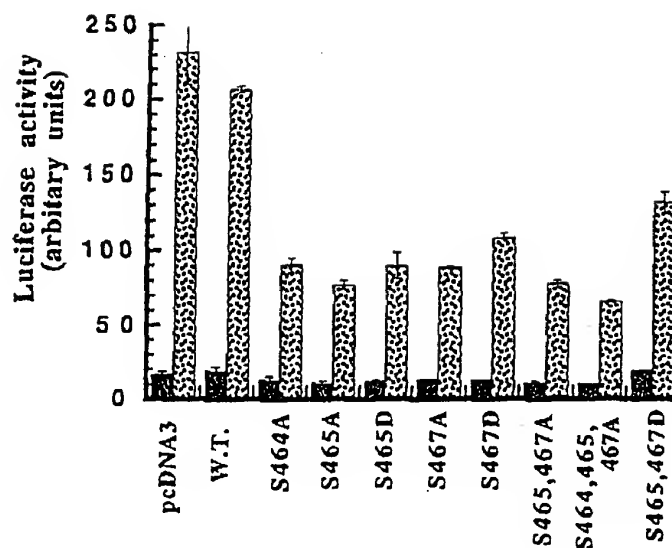


FIG.

5

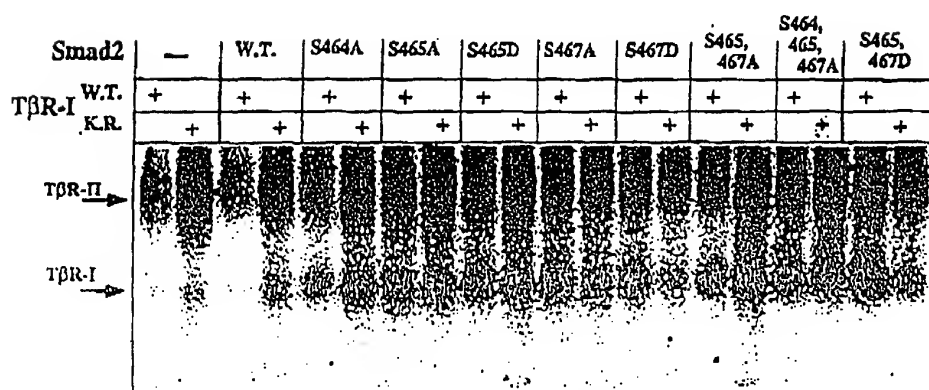
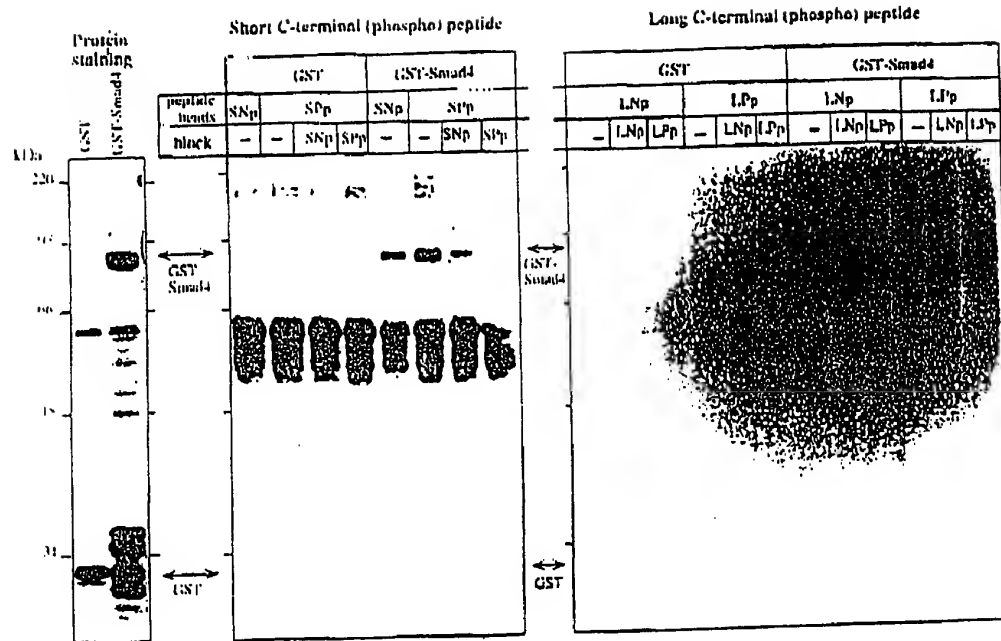


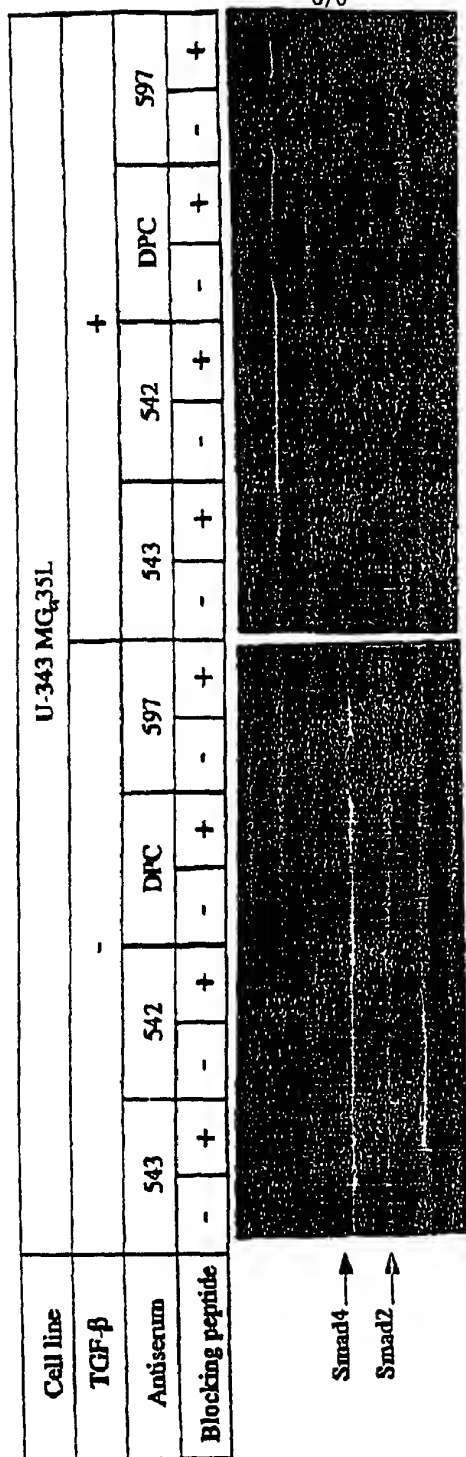
FIG. 6



TGF- β	-	+
T β R-II/T β R-I	+	
Smad2	+	
Smad7	-	0.1 μ g 1 μ g

← Smad2

Fig. 7

*Fig. 8*

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 98/10271

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 6	C12N15/12	C12N5/10	C07K14/47	C07K16/18	G01N33/50
	G01N33/53	G01N33/577	G01N33/68	A61K38/17	A61K39/395
According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED					
Minimum documentation searched (classification system followed by classification symbols)					
IPC 6 C12N C07K G01N A61K					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	MACIAS-SILVA M ET AL: "MADR2 is a substrate of the TGF-beta receptor and its phosphorylation is required for nuclear accumulation and signalling" CELL, vol. 87, 27 December 1996, pages 1215-1224, XP002077767 cited in the application				1-13, 21-30, 42-46
Y	see the whole document				14-17
Y	LAGNA G ET AL: "Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways" NATURE, vol. 383, 31 October 1996, pages 832-836, XP002077768 cited in the application see figure 4				14-17
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.					
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family					
Date of the actual completion of the international search			Date of mailing of the international search report		
18 September 1998			01/10/1998		
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016			Authorized officer Lonnoy, O		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10271

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NAKAO A ET AL: "Identification of Smad2, a human Mad-related protein in the Transforming Growth Factor beta signaling pathway" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 5, 31 January 1997, pages 2869-2900, XP002077775 cited in the application see figures 2,3D ---	18-20, 42-46
P,X	WO 98 07849 A (SCHERER W STEPHEN ;WRANA JEFFREY (CA); HSC RES DEV LP (CA); ATTISA) 26 February 1998 see the whole document ---	1-49
P,X	SOUCHELNYTSKYI S ET AL: "Phosphorylation of Ser-465 and Ser-467 in the C-terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signalling" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 44, 31 October 1997, pages 28107-28115, XP002077769 see the whole document ---	1-30, 42-46
P,X	ABDOLLAH S ET AL: "TbetaRI Phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 272, no. 44, 31 October 1997, pages 27678-27685, XP002077770 see the whole document ---	1-17, 21-30, 42-46
A	WRANA J AND ATTISANO L: "MAD-related proteins in TGF-beta signalling" TRENDS IN GENETICS, vol. 12, no. 12, December 1996, pages 493-496, XP002077771 ---	
A	CHEN X ET AL: "A transcriptional partner for MAD proteins in TGF-beta signalling" NATURE, vol. 383, 24 October 1996, pages 691-696, XP002077772 cited in the application ---	
A	KRETZSCHMAR M ET AL: "The TGF-beta family mediator Smad1 is phosphorylated directly and activated by the BMP receptor kinase" GENES AND DEVELOPMENT, vol. 11, no. 8, 15 April 1997, pages 984-995, XP002077773 -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/10271

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 21-23, 25, 26 and 47-49 are directed to methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/10271

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9807849 A	26-02-1998	AU 3936497 A	06-03-1998